THE UNIVERSITY OF ALBERTA

THE SPECIFICITY OF HELPER FACTOR IN THE CYTOTOXIC T CELL RESPONSE

by

© ALEXANDER W. PROCYSHYN

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE
IN
EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

EDMONTON, ALBERTA
FALL, 1978

To

my wife, Mina

and my parents, John and Irene

Abstract

The questions of genetic restriction and alloantigen specificity of helper factor in the cytotoxic T cell response were investigated. The generation of helper factor by mixed lymphocyte reaction-primed cells in the presence of 2-mercaptoethanol was found to be T cell-dependent. It was also found that although the I-A subregion of the H-2 complex is important in the control of its generation, helper factor could also be demonstrated in mixed lymphocyte-reactive cells responding to H-2 differences other than I-A. Helper factor generated after two days of priming was neither genetically restricted nor alloantigen specific. However, this method of helper cell generation may have led to polyclonal activation of helper cells of different specificities, resulting in the observed finding that it is not alloantigen specific. It was therefore attempted to isolate an enriched population of specific alloantigen-reactive cells which could be used as a source of helper factor generation. This population could not be isolated by cell size fractionation, but isolation was achieved by repeated stimulation of mixed lymphocyte-reactive cells. However, thorough studies on helper factor generation by these enriched specific alloantigen-reactive cells have not yet been performed.

ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to:

Dr. Kwok-Choy Lee for his invaluable assistance in this study and preparation of the manuscript. His unlimited patience and kindness was exemplary.

Miss Mabel Wong for her expert technical assistance and the attitude she displayed which made the most menial tasks a pleasure to perform.

Dr. H.T.G. Williams who inspired this study.

Dr. E. Diener for his help in arranging this study.

Ms. Iris Dyer who kindly typed the manuscript and whose assistance was invaluable in the technical preparation of this manuscript.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I. Introduction

A. History

The phenomenon of immunity was observed as early as the time of Thucydides, who wrote that the plaque would have claimed far more lives but for the devotion to nursing the ill by those who had recovered from the disease, since it was known that no one ever suffered from it a second time (1,2). It was probably similar observations of natural immunization that led, by the fifteenth century, to the Chinese practice of variolization (1,2). However, it was not until 1798 that Edward Jenner demonstrated that immunization against smallpox could be accomplished by using non-virulent inoculation of material from cowpox lesions (1). Louis Pasteur's development of the germ theory extended the knowledge and practice of vaccination (2).

By the late nineteenth century, it was demonstrated that immunity to some bacterial infections was due to the presence of humoral substances that became referred to as "antibodies." Metchnikoff challenged the view that humoral substances were solely responsible for immunity, and in 1883, demonstrated that phagocytes play an important role in resistance to disease (1,2). With his observations, a cellular component in the immune response gradually became established. Yet, it was not until 1942 that an immune response evoked in the absence of antibody formation could be demonstrated (1). This response ultimately became known as "delayed-type hypersensitivity." It was shown that it was accompanied by a cellular infiltration in which mononuclear phagocytic cells were very much in evidence, and that this state of sensitivity could be transferred to normal animals by lymphoid cells obtained from

highly sensitized donors (1,2). These observations led to the establishment of the lymphocyte as a key cell in the immune response.

In 1955, it was conclusively demonstrated that plasma cells can secrete antibody and that they arise from the differentiation of lymphocytes (1,2,3).

B. Classification of Lymphocytes

The study of the cells involved in the immune response led to observations of lymphocyte maturation. In birds, it became clear that the Bursa of Fabricius was essential for the development of lymphocytes that could differentiate into antibody-secreting plasma cells (4). As mammals do not have a Bursa of Fabricius, a bursa-equivalent was postulated, and although its anatomical site is as yet undetermined, the bone marrow became a popular proposed site (5-7). The thymus was demonstrated to be essential for the maturation of lymphocytes which could mediate a variety of immune responses in the absence of antibody formation (cell-mediated immunity) (5,8-12). Thus, immunocompetent lymphocytes were categorized into bone marrow-derived (bursa-derived in fowl), responsible for humoral immunity, and thymus-derived, responsible for cell-mediated immunity. These cells have subsequently become termed B lymphocytes (B cells) and T lymphocytes (T cells) (13), respectively.

Studies of surface membrane alloantigen markers (14-17) and the morphology (electron microscope) of mitogen-stimulated lymphocytes (17) have further distinguished these two classes of lymphocytes beyond their functional and ontogenic differences (Table I).

It has become clear that T cells mediate a variety of different functions (Table II) and recent studies suggest that functional subsets

		T cells	B cells
A.	Surface Antigen		
	1. Thy 1 (θ) (14)	positive	negative
	2. Immunoglobulin (17)	negative	positive
	3. Ly (15,16)	positive	negative
B.	Peripheral Localization (18) (as % of lymphocytes)		
	1. Blood	90% or more	10% or less
	2. Thoracic duct	80-85%	15-20%
	3. Lymph nodes	75%	25%
	4. Spleen	35-40%	60-65%
C.	Electron Microscopy (17)		
	1. Cell Surface	bland	rough
	2. Quantity of Rough Endoplasmic Reticulum	virtually none	abundant

Table I Cell Surface Antigen and Anatomical Differences Between T and B Lymphocytes

A. B Cells

1. Antibody-forming cells (4,8)

2. B memory cells (19)

B. T Cells

1. Delayed-type hypersensitivity-reactive cells (11,20,21)

2. Cytotoxic (killer) cells (22-25)

3. Gravt-vs-host-reactive cells (26-28)

4. Helper cells (10,26-32)

5. Mixed lymphocyte-reactive cells (33-38)

6. Suppressor cells (39-41)

C. Other Lymphocytes

1. Null cells (42)

2. Natural killer cells (43-45)

3. Antibody-dependent cytotoxic cells (46)

Table II A Classification of Lymphocytes Based on Effector Functions that can be Assayed

of T cells may also be distinguished by their phenotypic expression of surface markers (15,16).

More recently, other categories of lymphocytes which do not conform to the characteristics of T or B cells have been described. They have been called null cells (42), natural killer cells (43-45) and antibody-dependent cytotoxic cells (46). However, their in vivo functional significance has not yet been elucidated.

C. Cellular Cooperation in the Immune Response

Although B cells have been definitely established as the only producers of antibody, animals deprived of T cells (e.g. by neonatal thymectomy) are incapable of producing normal amounts of antibody against most antigens, and responsiveness can be restored by a source of T cells [e.g. thoracic duct lymphocytes (9,12)] (5-7,10). Thus, T-B cell cooperation is a requirement for a normal antibody-forming response to a variety of antigens. This cooperative interaction, however, is not a universal phenomenon. Some antigens (mostly polymers) are able to stimulate a normal antibody-forming response in the apparent absence of T cells, and have been called "T-independent antigens" (47,48).

In parallel to T-B cell interaction, cooperation between subsets of T cells (i.e. T-T interaction) has also been shown to be required for the induction of good cell-mediated responses (26,28,31,32,49).

In addition to lymphocytes, macrophages have been shown to be essential for the initiation of immune responses (50-55). The role of the macrophage appears to be to bind (56,57), process (58,59), and present (60) antigen to lymphocytes.

D. Helper T Lymphocytes

The subset of T cells which are essential in amplifying the effector functions of other lymphocytes have been named "helper T lymphocytes" (helper cells). Apart from their functional characteristics, helper cells can be distinguished from other lymphocytes.

1. Properties of Helper Cells

Helper cells bear the Thy 1 antigen on the cell membrane (61,62), as do all subsets of T cells (14). They also express the Ly 1^+ 2^- 3^- surface antigen phenotype (15,16,63,64), which distinguishes them from some other subsets of T cells (e.g. cytotoxic cells express Ly 1^- 2^+ 3^+). There is also evidence that some helper cells (involved in the antibody-forming response) express Ia surface antigen (65,66). Unlike many subsets of lymphocytes, helper cells are cortisone-resistant (67-69) and are radioresistant after activation (62,70). It has also been shown that proliferation of helper cells is essential before a helper effect can be observed (31).

2. Genetic Control of Helper Cell Activation

Immune responses to some antigens have been shown to be controlled by the major histocompatibility complex (MHC) (H-2 in the mouse) (2,71,72), which also codes for antigenic determinants expressed on cell surfaces (73). Studies of genetic recombinant mouse strains have defined various subregions of the H-2 complex (Figure 1).

Cell surface antigens coded for by the K and D regions are the classical serologically-defined antigens and serve as targets for cytotoxic T cells (73).

The I region contains enough DNA for many genes and controls a variety of immune phenomena including responsiveness to many antigens (71). There is some evidence that the antigens coded for by this

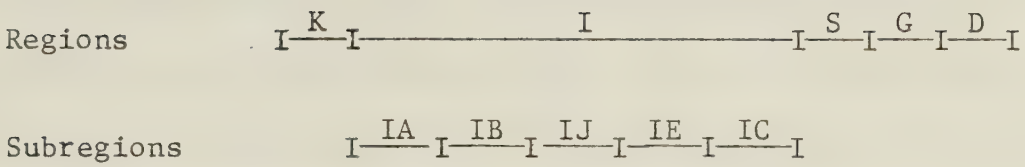

Figure 1. A diagrammatic representation of the mouse major histocompatibility (H-2) complex.

region, the I region-associated (Ia) antigens, can induce cytotoxic activity, although they are weaker cytotoxicity determinants than antigens coded for by the K and D regions (74-76).

The I region is also of significance in the mixed lymphocyte reaction (MLR) (77). T cell proliferation in MLR is most marked when I-A subregion incompatibilities exist between the responder and stimulator cells (15,16,75,78-80). As MLR-responsive T cells include helper cells (37), I-A subregion determinants appear to be important in the activation of helper cells.

More significantly, the I region regulates cooperative interactions between T and B cells (81-83). Helper T cells are stimulated by antigen in association with I region products (66,77,78,83).

3. Mechanism of Action of Helper Cells

The exact mechanism of action of helper cells has not yet been unequivocally established, but their requirement for both B and T cell-mediated immune responses is universally accepted. Helper cell activity was originally demonstrated in a T cell-deficient animal by the augmentation of the humoral response with T cells injected into the animal (7,10). Subsequently, T-B cell contact was shown to be unnecessary, as T-B cooperation can take place across a cell-impermeable membrane (84). Furthermore, the requirement for helper cells can be replaced by supernatants (61, 85-87) or homogenates (88) of activated helper cells. Thus, a helper cell-derived soluble factor has been proposed as the mediator of T-B cell cooperation (and helper-effector T cell cooperation). However, the molecular structure of helper factor has not yet been characterized, and attempts at doing so have led to inconsistent observations [e.g. molecular weight estimates range from under 10,000 daltons to 200,000 daltons (89-94)].

E. The Specificity of Helper Factor

1. The Problem

Perhaps the most perplexing question concerning helper factor is that of its specificity. The specificity question can be divided into two parts: firstly, "Can helper factor generated in one animal augment the immune response in an allogeneic animal?" and secondly, "Can helper factor generated in response to an antigen augment the immune response of the host to a second antigen?"

In examining the antibody-forming response, several investigators have found that helper factor activity could only be demonstrated if the helper cell population was syngeneic at the I region with the responding B cells (genetic restriction) (64,84,88,95). This genetic restriction appears to be related to the presence of an I region product (Ia antigen) on the helper factor which can be removed by anti-Ia immunoadsorbant columns (96,97).

However, helper factor which augments the B cell response but is not genetically restricted has also been described (61,98). This helper factor also bears Ia antigen (66,99) as well as other antigen, β_2-microglobulin (100).

The question of antigen specificity of helper factor has also been in dispute. The B cell response to an antigen has been found to be augmented only by helper factor generated by priming helper cells to the same antigen (64,83,84,87,88,101-103). However, using different experimental conditions, helper factor which is nonspecific with respect to antigen has also been generated (85,86,104-107).

Disparities similar to those seen in studies of specificity of helper factor in the humoral response are also seen in studies of its specificity in T cell-mediated responses. For the interaction

between helper and cytotoxic T cells, there is no genetic restriction (62,108). But as in the humoral response, helper activity augmenting the cytotoxic T cell response has been found to be both antigen specific (109) and nonspecific (110-112).

Thus, the question of specificity of helper factor in augmenting the cytotoxic T cell response remains unresolved.

2. The Approach to the Problem

Both genetic restriction and antigen specificity of helper factor in the cytotoxic T cell response were examined.

The experimental system chosen to assay for helper factor activity was one which separates helper cells from the responding cytotoxic cells by a cell-impermeable membrane (69,84). It allows for an *in situ* production of helper factor which crosses the membrane in a solution which is continuous with the responding cytotoxic cell population. As the magnitude of the cytotoxic response is dependent on the dose of responding cells (24), using small numbers of responders will limit cytotoxicity. The response can then be amplified by the addition of helper factor to the culture system.

This method of helper factor generation has a major advantage over that of the other system used to demonstrate helper factor activity, the supernatant system (111,112). Here, helper factor is harvested as the supernatant from activated helper cells and is added to the responding cell population. Thus, helper factor activity may be limited by length of time it can remain functional in tissue culture. Using a source of continually generated helper factor, as in the membrane system, can avoid this problem.

Helper cells were generated in MLR by immunizing (priming) lymphocytes against irradiated allogeneic stimulator cells.

In order to show that the production of helper factor is T cell-dependent, helper cells were treated with antiserum specific for T cells (anti-Thy 1) and complement before priming with stimulator cells.

The H-2 complex was also studied to determine which portion of it is necessary for generation of helper factor. This was done using congenic resistant strains of mice which are genetically identical except at defined portions of the H-2 complex.

Finally, an attempt was made to isolate specific alloantigen-reactive cells (78). This was done because the generation of helper factor from a population of helper cells of multiple specificities may result in production of multiple monospecific helper factors. Should this occur, helper factor activity would appear to be non-antigen-specific. Thus, if helper cells responding to only a single H-2 haplotype could be isolated, the problem of multiple monospecific helper factors could be avoided, and the helper factor generated by this enriched population would be ideal for the study of its antigen specificity.

Two systems were used to enrich for specific alloantigen-reactive cells. The first was fractionation of alloantigen-stimulated cells on the basis of their size [velocity sedimentation separation (113)] (78,114). Here, the rationale is based on the observation that stimulation of T cells results in an increase in their size (blastogenesis) which precedes division (115). Only cells specific for the stimulating alloantigen are expected to undergo blastogenesis and these can be

separated from the smaller nonresponding cells using this technique (78,114).

The second system used to isolate alloantigen-reactive cells responding to a single H-2 haplotype was that of repeated stimulation of MLR-reactive cells (115-117).

The principle is also based on the observation that stimulation of T cells in MLR leads to their proliferation. Thus, repeated stimulation by the same allogeneic cells should result in repeated proliferation of the cells responding to them. Moreover, stimulation is required to maintain prolonged lymphocyte survival in tissue culture (116) and therefore the nonresponding cells in MLR will die during the increased time that is used for restimulation. Thus, restimulation of MLR-reactive cells should result in great enrichment of the cells responding to the particular stimulating allogeneic cells.

Following isolation of stimulated cells using the two systems described, they were assayed for their specificity in proliferating in response to various allogeneic stimulator cells (MLC assay) as well as their specificity in the cytotoxic response. The MLC assay detects cell proliferation primarily in response to I region incompatibilities between responder and stimulator cells, while the cytotoxic activity is seen primarily in response to K and/or D region incompatibilities (80). Thus, both assays were done as they detect different determinants on alloantigens and therefore would allow for more reliable criteria to study the specificities of the alloantigen-reactive cells.

Chapter II. Materials and Methods

A. Mice

Eight to 16-week-old mice of both sexes were used. C57BL/10J (B10) mice and their congenic resistant strains on the B10 background, B10.D2/oSn, B10.AKM/Sn, and B10.A(5R), which are genetically identical except at the MHC, were obtained from the Jackson Laboratory, Bar Harbor, Maine, as was the C57BL/6 strain. Other B10 congenic resistant strains, B10.A(2R), B10.A(4R), and B10.T(6R), as well as the AQR strain, were obtained from Dr. Fritz Bach, the University of Wisconsin School of Medicine, Madison, Wisconsin. These strains as well as CBA/CaJ, DBA/2J, C3H.HeJ, C3H.OH, C3H.NB/Sn and AKR/J production breeders from the Jackson Laboratory were bred at the University of Alberta Health Sciences Animal Center, Edmonton, Alberta. The MHC haplotypes of the mouse strains are listed in Table III.

B. Medium

Unless otherwise specified, the medium used was RPMI 1640 (Gibco Canada Ltd., Calgary, Alberta), supplemented with 3.5 g of $NaHCO_3$/1, 10,000 units Penicillin and 10 mg Streptomycin/1 (Gibco Canada), and 10% (v/v) fetal calf serum (FCS) (Reheis Chemical Company, Phoenix, Arizona, or Gibco Canada).

For the assay of cell proliferation in mixed lymphocyte culture (MLC), human serum was used in lieu of FCS. The blood for human serum was obtained by venipuncture of volunteers and allowed to clot at room temperature. The clot was allowed to retract overnight at 4°C. The blood was then centrifuged at 800 xg for 20 min and the serum was collected, heated in a water bath at 7°C for 30 min to inactivate complement, and sterilized by passage through a 0.45 μm pore size filter (Nalgene

Strain	Haplotype	H-2 subregions K	I-A	I-B	I-J	I-E	I-C	S	G	D
C57 BL/10J	$H\text{-}2^{b}$	b	b	b	b	b	b	b	b	b
B10.D2/oSn	$H\text{-}2^{d}$	d	d	d	d	d	d	d	d	d
B10.AKM/Sn	$H\text{-}2^{m}$	k	k	k	k	k	k	k	k	q
B10.T(6R)	$H\text{-}2^{y2}$	q	q	q	q	q	q	q	?	k
B10.A(2R)	$H\text{-}2^{h2}$	k	k	k	k	k	d	d	d	d
B10.A(4R)	$H\text{-}2^{h4}$	k	k	b	b	b	b	b	b	b
B10.A(5R)	$H\text{-}2^{i5}$	b	b	b	k	k	d	d	d	d
C57BL/6	$H\text{-}2^{b}$	b	b	b	b	b	b	b	b	b
AQR	$H\text{-}2^{y1}$	q	k	k	k	k	d	d	d	d
AKR/J	$H\text{-}2^{k}$	k	k	k	k	k	k	k	k	k
CBA/CaJ	$H\text{-}2^{k}$	k	k	k	k	k	k	k	k	k
DBA/2J	$H\text{-}2^{d}$	d	d	d	d	d	d	d	d	d
C3H.HeJ	$H\text{-}2^{k}$	k	k	k	k	k	k	k	k	k
C3H.OH	$H\text{-}2^{o2}$	d	d	d	d	d	d	d	d	k
C3H.NB/Sn	$H\text{-}2^{p}$	p	p	p	p	p	p	p	p	p

Table III Mouse Strain H-2 Complex Haplotypes (72)

filter unit, Sybron Corporation, Rochester, New York). It was then stored in aliquots at -70°C until needed.

C. Tumor Cells

P815 mastocytoma cells ($H\text{-}2^d$, originated from a DBA mouse) were used. They were grown in tissue culture in RPMI 1640 (Gibco Canada) supplemented with 10% FCS (Gibco Canada) and 50 mg Geutamycin/l (Schering Corporation, Kenilworth, New Jersey). Once every three months, these cells were passaged through syngeneic mice (DBA/2J), and new stock cultures were started from these cells. One day before cells were required for an assay, new cultures were set up at a concentration of $2x10^5$/ml. This ensured the availability of expotentially growing cells for the assay (118).

D. Preparation of Cells for Tissue Culture

Mouse spleen cells were used as the source of lymphocytes. Mice were killed by cervical dislocation and spleens were removed and collected in Puck's saline. The spleens were minced with scissors and forced through a 200 gauge stainless steel wire mesh. The cell suspension was collected in plastic tubes (Falcon 2001 tubes, Falcon Plastics, Oxnard, California) and thoroughly suspended with a 5 ml pipette. Clumps were allowed to settle for 5 min and the fluid containing the cells was centrifuged at 250 xg for 7-1/2 min. The supernatant fluid was then aspirated off and the cell pellet was suspended in medium. Viable cells were counted on a hemocytometer.

E. Generation of Helper Cells

All helper cells were generated in tissue culture from mouse spleen cells stimulated with allogeneic spleen cells. Each culture contained

$4x10^6$ responder cells and $4x10^6$ irradiated (1500 rads from a ^{137}Cs source, Gamma Cell 40, Atomic Energy of Canada, Ltd.) stimulator cells in 2.5 ml of medium in 16 mm Linbro flat-bottomed trays (76-033-05, Flow Laboratories, Inglewood, California). The cultures were incubated at 37°C in an atmosphere of 10% CO_2 in air with >96% humidity.

F. Assay of Cell Proliferation in Mixed Lymphocyte Culture (MLC)

Proliferation of cells in MLC was measured by their uptake of tritiated thymidine. Triplicate cultures containing various numbers of responder cells and a constant number ($1x10^6$) of irradiated stimulator cells in 0.3 ml of RPMI with 10% (v/v) human serum per well were set up in Linbro 96 well flat-bottomed trays (76-003-05, Flow Laboratories, Inglewood, California). The cultures were incubated in a humidified (>96%) atmosphere of 10% CO_2 in air at 37°C for 3 days. Each well was then pulsed with 0.5 µCi [methyl-^3H] thymidine (2 Ci/mmole, Amersham Corporation, Oakville, Ontario) in 0.02 ml of medium. After a further incubation period of 20 h, the cultures were harvested onto glass fiber filters with a Titretek Cell Harvester (Flow Laboratories, Inglewood, California) and washed with distilled water. The filters were dried in an oven and placed in plastic vials (Bio-vials, Beckman Instruments, Irvine, California) containing 1 ml aliquots of scintillation fluid. Radioactivity was determined by a Packard Tricarb liquid scintillation spectrometer (Packard Instrument Company, Downer's Grove, Illinois). The results are expressed as counts per minute (cpm) per culture $\pm$ the standard deviation.

G. Cytotoxicity Assay

Cytotoxic activity of cells in tissue culture was assayed by their ability to lyse ^{51}Cr-labelled target cells (119). The requirements for appropriate target cells are i) susceptibility to lysis, ii) large size and hence greater sensitivity, iii) high uptake of ^{51}Cr, and iv) low background chromium release (24). For this purpose, large tumor cells such as P815 mastocytoma cells ($H\text{-}2^d$, syngeneic to DBA/2 mice) are suitable. However, many strains of mice do not have tumors suitable for the chromium release assay, and therefore lymphoid cells are used as targets. Since lymphoid cells are small, they have to be stimulated to differentiate into large lymphoblasts. This was done by culturing spleen cells (6.7×10^6/ml) in medium containing 5×10^{-5}M 2-mercaptoethanol (J.T. Baker Chemical Company, Phillipsburg, New Jersey) and 2 µg Concanavalin A (Con A)/ml (Calbiochem Laboratories, San Diego, California). The blasts were used two days later. They were radiolabelled by incubating 5×10^6 cells in 0.5 ml medium containing 0.2 mCi $Na_2{}^{51}CrO_4$/ml (New England Nuclear, Boston, Massachusetts) for 1 h at 37°C. The labelled cells were washed three times with medium.

Microcultures to be assayed for cytotoxicity which could not be conveniently harvested were assayed *in situ*. 0.1 ml of supernatant (of the 0.3 mls in each microculture) was aspirated from each well and replaced with 0.1 ml medium containing 2×10^4 ^{51}Cr-labelled Con A blasts. These cultures were reincubated for 4-1/2 h, at which time 0.15 ml of supernatant fluid was collected from each well in glass tubes (12 x 75 mm, Corning Glass Works, Corning, New York) and radioactivity was determined by a Beckman Gamma 300 radiation counter (Beckman Instruments, Irvine, California).

Tissue culture cells (in triplicate) which could be conveniently harvested were pooled in 0.4 ml medium. Six serial two-fold dilutions were performed in duplicate in Linbro V-bottomed tissue culture trays (76-023-05, Flow Laboratories, Inglewood, California), such that each well contained harvested cells in 0.1 ml medium. $2x10^4$ ^{51}Cr-labelled target cells in 0.1 ml medium was added to each well. The trays were incubated at 37°C for 4-1/2 h, after which time 0.1 ml of supernatant fluid was collected for determination of radioactivity.

Cytotoxic activity was expressed as percentage of specific lysis according to the formula (119):

$$\% \text{ specific lysis} = \frac{\text{experimental counts - spontaneous counts}}{\text{total counts - spontaneous counts}} \times 100\%$$

Total counts were defined as the radioactivity released from ^{51}Cr-labelled target cells in the presence of 0.05% (v/v) Zap-isoton (Coulter Electronics, Mississauga, Ontario). Spontaneous counts were defined as the radioactivity released by target cells incubated alone, or in the presence of spleen cells from an unimmunized culture.

H. Assay for Helper Factor Activity

Modified Marbrook double chambers (69,84) were used to determine helper factor activity in augmenting a cytotoxic T cell response. This culture system allows helper factor to be produced *in situ* and passed in solution across a cell-impermeable membrane to limiting numbers of responder cells which, in the absence of helper factor, cannot support a cytotoxic response.

The culture chambers were constructed such that the responder cells rested on a thoroughly washed dialysis membrane (Fisher Scientific Company, Pittsburgh, Pennsylvania) fixed by a sleeve of silastic tubing (Dow-Corning Corporation, Midland, Michigan) to a glass tube 1.1 cm in

diameter. The helper cells rested on a cell-impermeable Metricel membrane (Type GA-6, pore size 0.45 μm; Gelman Instrument Company, Ann Arbor, Michigan), which was glued (Silastic Medical Adhesive, Silicone type A, Dow Chemical Corporation, Midland, Michigan) onto the end of the inner glass chamber (0.8 cm in diameter). The volumes of medium in the inner and outer chambers were 0.6 ml and 0.5 ml respectively. The distance separating the two cell populations was 2 mm, and at this distance, the medium in each chamber was at the same level. The chambers were suspended in approximately 40 ml medium in a 100 ml flask.

All cultures were set up in triplicate. Limiting numbers of responder cells were mixed with a fixed number ($1x10^6$) of irradiated stimulator cells in the lower chamber. In the upper chamber, a range of concentrations of helper cells was stimulated with $1x10^6$ irradiated stimulator cells. 2-mercaptoethanol (2-ME) was added to all medium at a final concentration of $5x10^{-5}$ M. The cultures were incubated for 5 d at 37°C. Each group of cultures was then harvested, pooled and suspended in 0.4 ml medium for the cytotoxicity assay.

I. Anti-Thy 1 Treatment of Helper Cells

Anti-Thy 1 serum was raised in AKR/J mice by multiple intraperitoneal immunization with CBA/CaJ thymus cells (55). The specificity of this serum for T cells was demonstrated functionally by the abolition of the T-dependent antibody-forming response to sheep erythrocytes while leaving T-independent responses to DNP-ficoll and polymeric flagellin intact (55).

The helper cells were incubated at 10^7 cells/ml with anti-Thy 1 serum (concentration 1 in 40) in medium without FCS. After 45 min at 0°C, the cells were centrifuged (250 x g), suspended at $5x10^6$ cells/ml of agarose-adsorbed guinea pig complement (1 in 6 in medium without FCS), and re-

incubated at 37°C for 30 min. The cells were then washed 3 times to remove complement.

J. Velocity Sedimentation Gradient Separation of Spleen Cells

Separation of cells on the basis of size by velocity sedimentation at unit gravity was performed at 4°C in a "Sta-put" apparatus (17.5 cm in diameter) as described by Miller and Phillips (113). All surfaces in contact with cells were siliconized (Siliclad; Becton, Dickinson and Company, Parsippany, New Jersey). A buffered step gradient of FCS (5 to 30%) in phosphate-buffered saline was used to stabilize the layers of separated cells. Spleen cells were thoroughly suspended in medium and loaded on the Sta-put ($3x10^8$ cells in 30 mls). After a sedimentation time of 4 h, the cone volume of 250 mls was collected and discarded. Then 15 ml fractions (38 altogether) were collected and the cell number in each fraction was determined by a Coulter Counter (Model B, Coulter Electronics, Hialeah, Florida). Sedimentation velocities were calculated as described by Armstrong and Kraft (120).

Chapter III. Results

A. The Generation of Helper Factor in Modified Marbrook Double Chamber Cultures

Cultures containing limiting numbers of CBA/CaJ ($H\text{-}2^k$) responder spleen cells developed minimal cytotoxic activity when stimulated by P815 mastocytoma cells (Figure 2). However, the addition to the upper chamber of the culture system of CBA/CaJ helper cells which had been primed for 2 days with C57BL/6 ($H\text{-}2^b$) or to (CBA/CaJ x DBA/2J)F_1 ($H\text{-}2^{k/d}$) spleen cells, resulted in a good cytotoxic response. These results indicate that helper factor is generated by the cells in the upper chamber and crosses the cell-impermeable membrane to cooperate with the responding cytotoxic cells in the lower chamber.

In addition, $H\text{-}2^k$ helper cells stimulated by $H\text{-}2^b$ and $H\text{-}2^{k/d}$ alloantigens were able to augment the anti-$H\text{-}2^d$ response, indicating that helper factor is not specific to the priming alloantigen.

B. Helper Factor Production is T cell-dependent

CBA/CaJ helper spleen cells were treated with anti-Thy 1 serum and complement, and control CBA/CaJ helpers were treated with normal mouse serum (NMS) and complement prior to the 2 days of priming to DBA/2J stimulator cells. The results show that the helper effect is abolished by pretreatment of helper cells with anti-Thy 1 serum and complement (Figure 3). As the Thy 1 antigen is borne on T cells but not on B cells, these results establish that helper factor production is T cell-dependent.

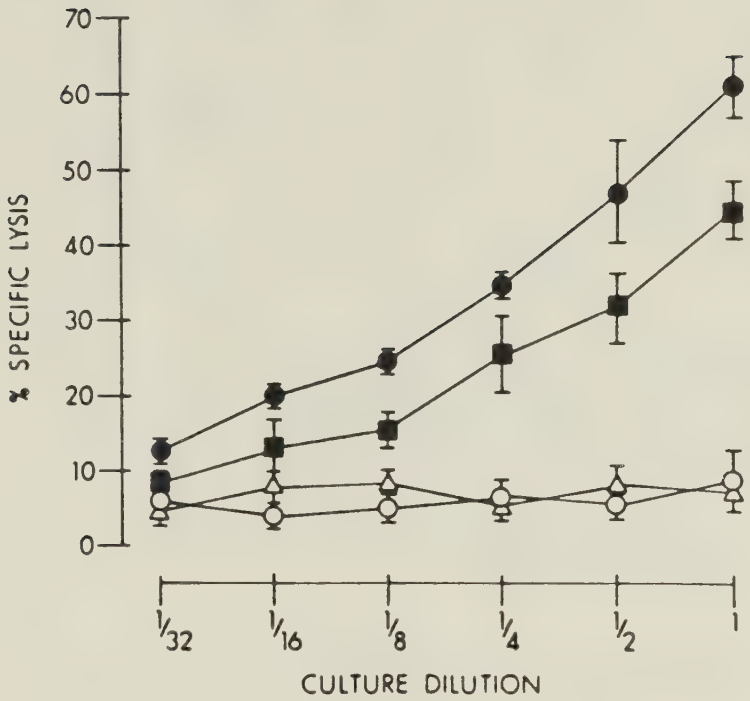

Figure 2. Generation of helper activity in modified Marbrook double chamber cultures. Cytotoxic cells were generated by stimulating $1x10^5$ CBA/CaJ spleen cells with $1x10^6$ P815 mastocytoma cells. Helper cells had been generated by stimulating CBA/CaJ spleen cells ($2x10^6$/well) with (CBA x DBA)F_1 or C57BL/6 spleen cells ($4x10^6$/well) for 2 days. Helpers ($5x10^5$), together with more stimulator cells ($1x10^6$), were added to the upper chambers and the cultures were incubated for 5 days: o - o no helpers; Δ - Δ unimmunized (CBA/CaJ stimulated by irradiated CBA/CaJ) helpers; • - • CBA anti-(CBA x DBA)F_1 helpers; ■ - ■ CBA anti-C57BL/6 helpers.

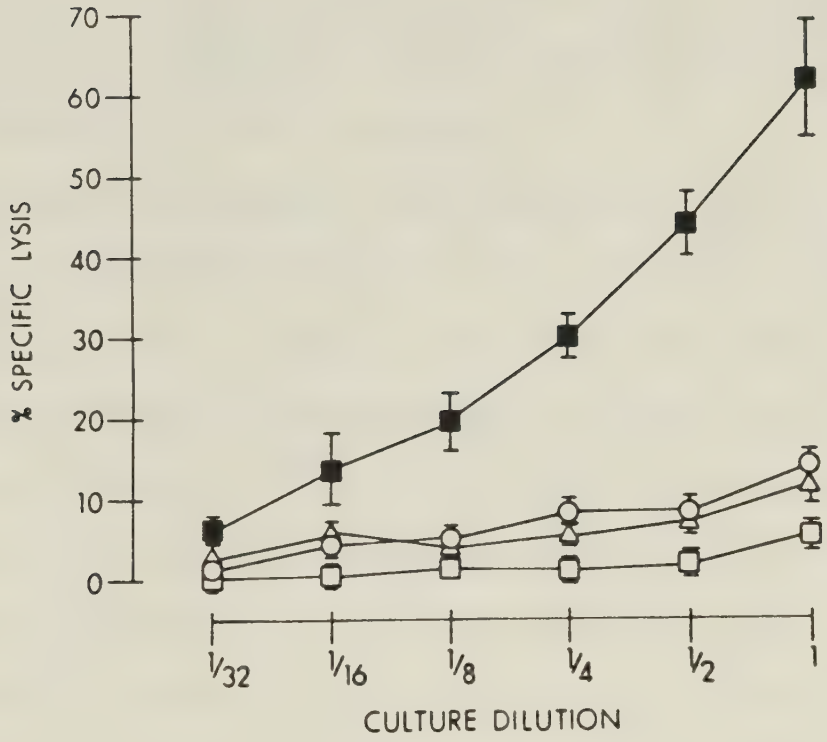

Figure 3. Helper factor activity is T cell-dependent. Cytotoxic cells were generated by stimulating $1x10^5$ CBA/CaJ spleen cells with $1x10^6$ P815 mastocytoma cells. Anti-Thy 1 serum plus complement-treated and NMS plus complement-treated CBA/CaJ spleen cells ($2x10^6$/well) were stimulated by irradiated DBA/2J spleen cells ($4x10^6$/well) for 2 days and used as helpers at $5x10^5$/culture with 10^6 irradiated DBA spleen cells. Cultures were incubated for 5 days: o - o no helpers; Δ - Δ unimmunized helpers; ■-■ NMS plus complement-treated helpers; □-□ anti-Thy 1 plus complement-treated helpers.

C. Genetic Requirements for Helper Factor Generation

The B10 anti-P815 cytotoxic response was studied. Helper cells were generated by MLR stimulation of several mouse strains in response to defined differences in the H-2 complex.

The results show that the most marked enhancement of the B10 anti-P815 cytotoxic response occurred with helper factor generated from mouse strains totally different at the H-2 complex (B10 anti-B10.D2) (Figure 4). Less prominent helper activity was generated when the mouse strains differed at only the I and S regions [AQR anti-B10.T(6R)], and this activity was further diminished if the strain differences did not include the I-A subregion [B10 anti-B10.A(5R) and B10.A(2R) anti-B10.A(4R)]. Thus, although I-A subregion differences in the H-2 complex are probably the most important in the generation of helper factor, other subregions are also involved to a lesser degree.

These results also demonstrate that helper factor generated from a variety of mouse strains stimulated by a variety of different allogeneic cells are able to enhance the B10 anti-P815 cytotoxic response. This suggests that helper factor is neither genetically restricted nor antigen specific.

D. Time Course for Blastogenesis

In order to undertake isolation of specific alloantigen-reactive cells, it was necessary to determine the time of peak blastogenesis (for the velocity sedimentation separation procedure) and the duration of T cell proliferation in MLR (for the enrichment by restimulation procedure).

A CBA/CaJ ($H\text{-}2^k$) anti-DBA/2J ($H\text{-}2^d$) response was studied. It was found that peak [^{3}H]-thymidine incorporation occurred on the 5th day of primary stimulation (Figure 5). By the 7th day after stimulation, [^{3}H]-

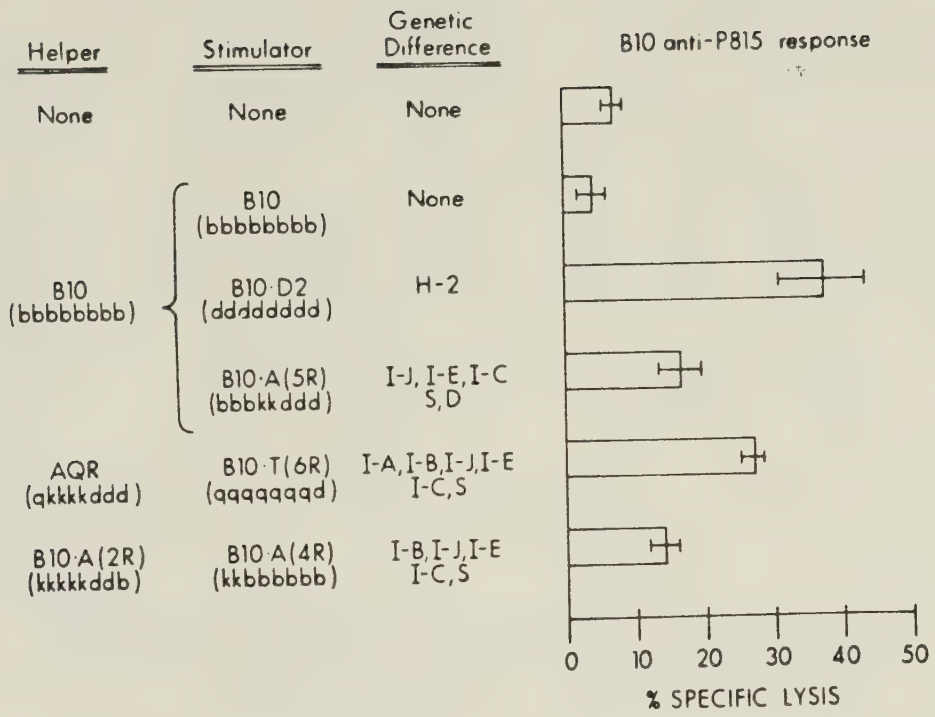

Figure 4. Genetic requirements for helper factor generation. Cytotoxic cells were generated by stimulating $2x10^5$ B10 spleen cells with $1x10^6$ P815 mastocytoma cells. Helper cells had been generated from several responder spleen cell strains ($2x10^6$/well) stimulated by several strains of irradiated spleen cells. Helper cells were used at $5x10^5$/culture plus $1x10^6$ stimulator cells. Cultures were incubated for 5 days. Noncapitalized letters represent individual H-2 subregion haplotypes. G region haplotypes are not shown.

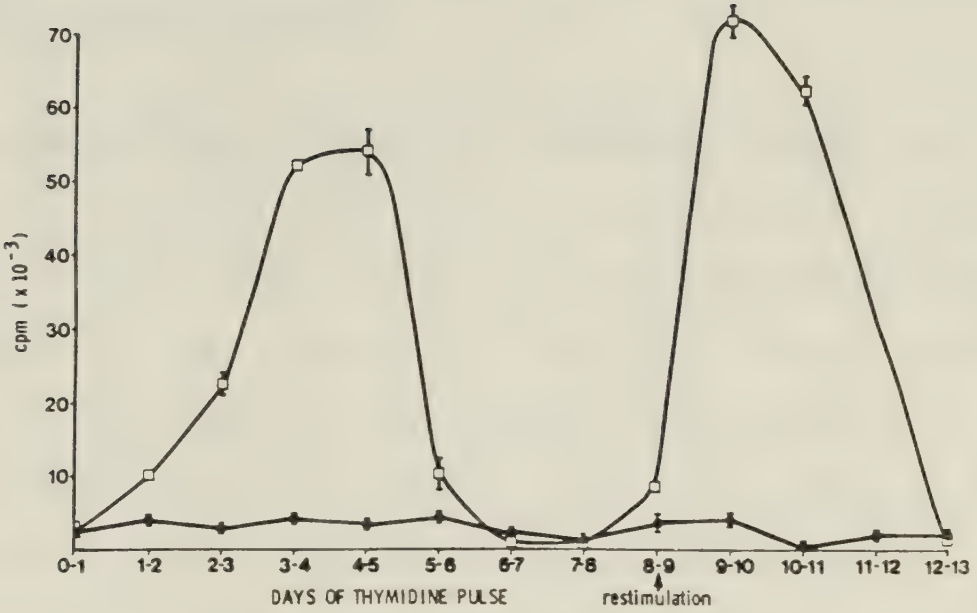

Figure 5. Time course for blastogenesis. CBA/CaJ spleen cells ($4x10^6$/well) were challenged with DBA/2J or irradiated (control) CBA/CaJ spleen cells ($4x10^6$/well) in 16 mm trays. Each day, triplicate cultures were pulsed with [^{3}H]-thymidine and after 20 h incubation, cells were suspended and 0.3 ml of fluid from each well was harvested for the determination of radioactivity. On the eighth day, the cultures were restimulated with fresh irradiated stimulator cells: ● - ● CBA/CaJ stimulated by irradiated CBA/CaJ; □ - □ CBA/CaJ stimulated by DBA/2J.

thymidine incorporation reached baseline levels. However, after repeated stimulation with the same allogeneic cells, peak blastogenesis was reached within 24 hours and the magnitude of the MLC response exceeded that induced by primary stimulation.

E. Velocity Sedimentation Separation of Alloantigen-reactive Cells

CBA/CaJ ($H\text{-}2^k$) responder spleen cells were stimulated by B10.T(6R) ($H\text{-}2^{y2}$) spleen cells in tissue culture. As peak blastogenesis occurs on the 5th day after primary stimulation (Figure 5), cell size separation was performed at this time. The separated cells were collected in 38 fractions and the cell number in each was determined (Figure 6). Fractions were then pooled into 3 groups with sedimentation velocities <1.5 mm/hr, 1.5-5.0 mm/hr, and >5.0 mm/hr (the blast fraction). The reactivity of these groups to alloantigens of various H-2 haplotypes was evaluated by the MLC and cytotoxicity assays. This was compared to the reactivity of unprimed CBA/CaJ responder cells and to primed but unfractionated CBA/CaJ responders. It was predicted i) that the unprimed cells would respond to all the stimulating alloantigens, ii) that primed cell responses would be of greater magnitude at lower concentrations than unprimed cells (Figure 5), and iii) that the blast fraction of the separated cells would respond specifically to B10.T(6R) (the priming alloantigen), as this fraction was enriched for responder cells reactive to B10.T(6R).

The results show, as predicted, that unprimed CBA/CaJ cells responded to several stimulating allogeneic cells (Figures 7 and 8). However, some strains of allogeneic cells appeared to be poor stimulators to CBA/CaJ responses.

As expected, primed CBA/CaJ spleen cells responded at lower concentrations than the unprimed cells (Figures 9 and 10). It is of interest

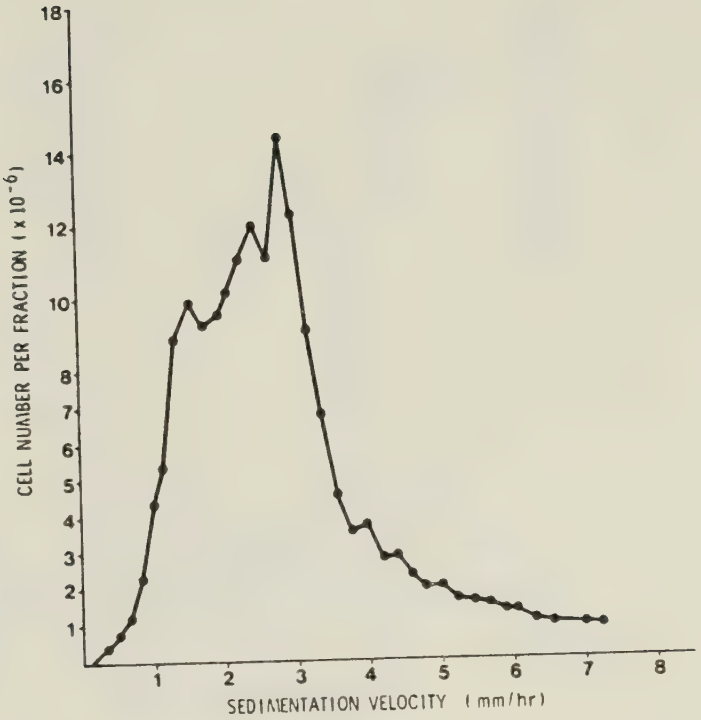

Figure 6. Velocity sedimentation profile of primed spleen cells. CBA/CaJ spleen cells ($4x10^6$/well) were primed with B10.T(6R) spleen cells ($4x10^6$/well) in 16 mm tissue culture trays for 5 days. $3x10^8$ responder cells were submitted to fractionation. $1.75x10^8$ cells were recovered after fractionation.

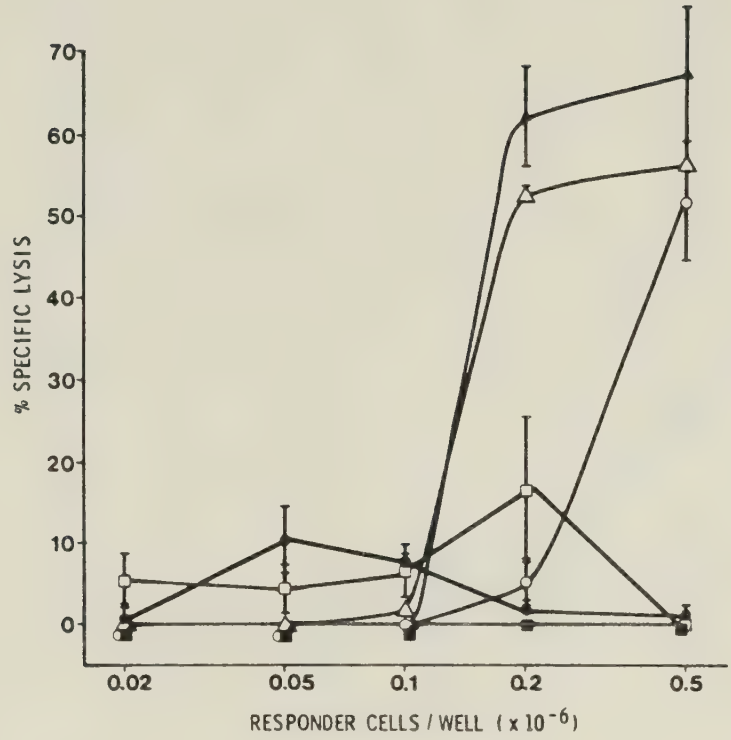

Figure 7. Cytotoxic response of unprimed spleen cells. Responder CBA/CaJ spleen cells were stimulated by several strains of allogeneic spleen cells ($1x10^6$/well) in 96 well flat-bottomed trays and incubated for 5 days, at which time an in situ cytotoxicity assay was performed using ^{51}Cr-labelled Con A blasts as target cells: ● - ● CBA/CaJ target cells (control); Δ - Δ B10.T(6R) targets; □ - □ C57BL/6 targets; o - o C3H.OH targets; ▲ - ▲ C3H.NB/Sn targets; ■ - ■ C3H.HeJ targets (H-2 identical with CBA/CaJ).

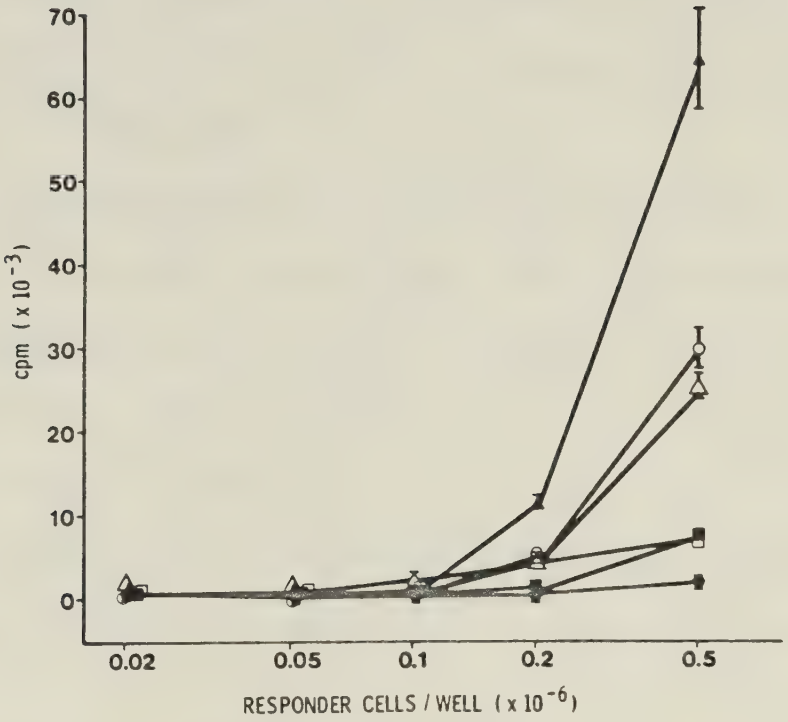

Figure 8. MLC of unprimed spleen cells. Responder CBA/CaJ spleen cells were stimulated by several strains of allogeneic cells ($1x10^6$) in 96 well flat-bottomed trays. After 3 days of incubation, the cells were pulsed with [^{3}H]-thymidine for 20 h; ● - ● CBA/CaJ stimulator (control); Δ - Δ B10.T(6R) stimulators; □ - □ C57BL/6 stimulators; o - o C3H.OH stimulators; ▲ - ▲ C3H.NB/Sn stimulators; ■ - ■ C3H.HeJ stimulators.

that the cytotoxic response of the primed-unfractionated cells occurred only when stimulated by B10.T(6R), the priming alloantigenic stimulator (Figure 9), yet MLC activity was seen in response to all the stimulating allogeneic cells (Figure 10).

Of the fractionated groups of CBA/CaJ cells, the group with sedimentation velocity <1.5 mm/hr (the smallest cell size fraction) elicited no cytotoxic response and no MLC activity against any of the allogeneic cells (Figures 11 and 12). This was unexpected because this fraction would be predicted to include responder cells with specificities for stimulator cells other than B10.T(6R). Thus, it would have been expected that this fraction would respond to all the stimulating cells except B10.T(6R).

The 1.5-5.0 mm/hr sedimentation velocity fraction showed no cytotoxic response (Figure 13) and poor MLC activity (Figure 14). However, the MLC activity occurs in response to all the stimulating allogeneic cells.

The blast fraction of CBA/CaJ responders primed to B10.T(6R) stimulators showed specific anti-B10.T(6R) cytotoxicity (Figure 15), as predicted. However, in MLC, the CBA/CaJ cells responded to all the stimulating allogeneic cells (Figure 16). This situation parallels that seen in the primed-unfractionated group (Figures 9 and 10). The MLC assay was viewed as more significant than the cytotoxicity assay for the purposes of isolating an enriched helper cell population, as helper cell proliferation parallels MLC activity, but not cytotoxicity (83,121).

As the blast fraction MLC responses were similar to primed-unfractionated cell responses and were not preferential to the priming allogeneic cells (except in magnitude), it was not possible to isolate an enriched pop-

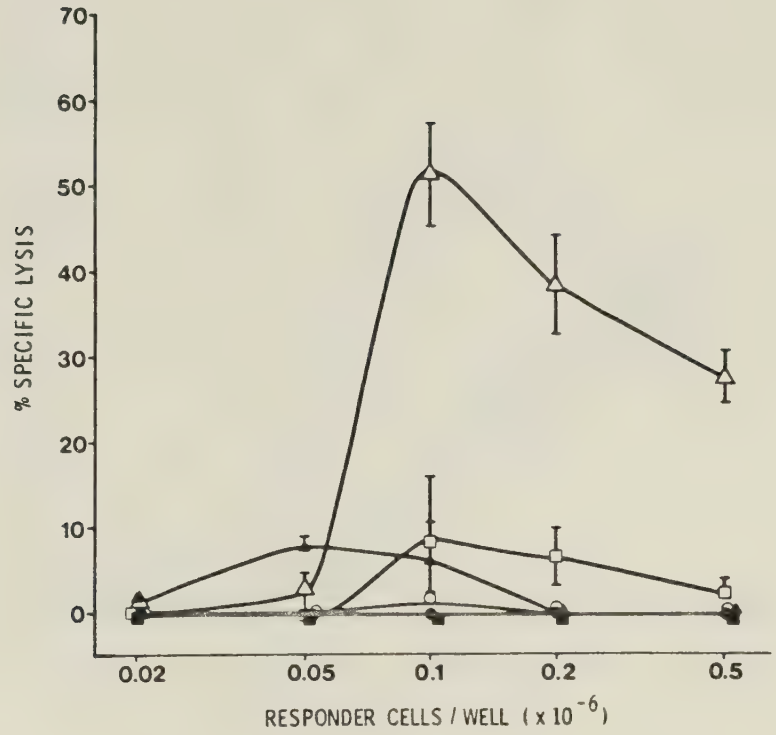

Figure 9. Cytotoxic response of primed spleen cells. CBA/CaJ spleen cells ($4x10^6$/well) were primed with B10.T(6R) spleen cells ($4x10^6$/well) in 16 mm culture trays for 5 days. These cells were then harvested and secondary cultures were set up with fresh stimulator cells (10^6/well) in 96 well trays for 5 days, at which time an in situ cytotoxicity assay was performed: ● - ● CBA/CaJ stimulators (control); Δ - Δ B10.T(6R) stimulators; □-□ C57BL/6 stimulators; o - o C3H.OH stimulators; ▲ - ▲ C3H.NB/Sn stimulators; ■ - ■ C3H.HeJ stimulators.

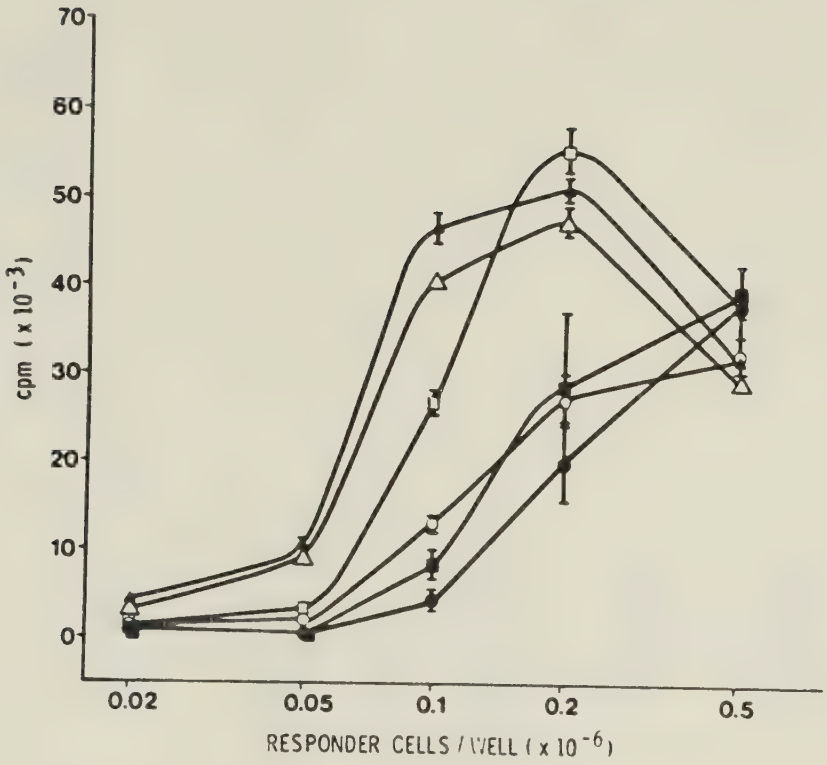

Figure 10. MLC of primed spleen cells. The experimental conditions were identical to those in Figure 9 except that the secondary cultures were pulsed with [^{3}H]-thymidine on the 3rd day for 20 h: ● - ● CBA/CaJ stimulators; Δ - Δ B10.T(6R) stimulators; □ - □ C57BL/6 stimulators; o - o C3H.OH stimulators; ▲ - ▲ C3H.NB/Sn stimulators; ■ - ■ C3H.HeJ stimulators.

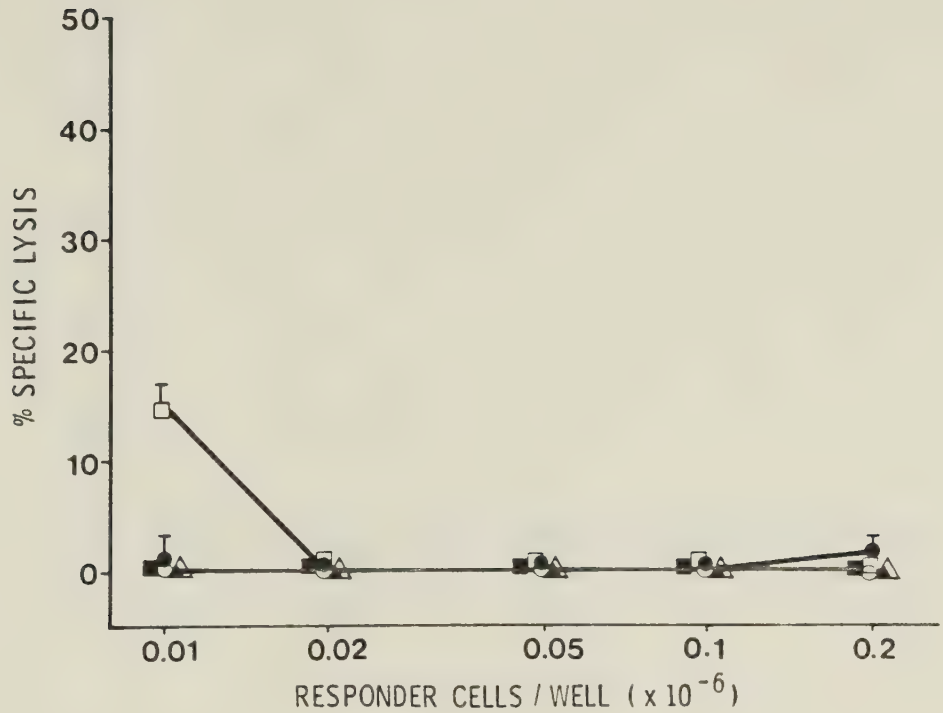

Figure 11. Cytotoxic response of the <1.5 mm/hr sedimentation velocity fraction of primed spleen cells. Fractionated cells were challenged with various strains of stimulator cells ($1x10^6$/well) in 96 well trays for 5 days, at which time an in situ cytotoxicity assay was done: ● - ● CBA/C-J stimulators; Δ - Δ B10.T(6R) stimulators; □-□ C57BL/6 stimulators; o - o C3H.OH stimulators; ▲ - ▲ C3H.NB/Sn stimulators; ■-■ C3H.HeJ stimulators.

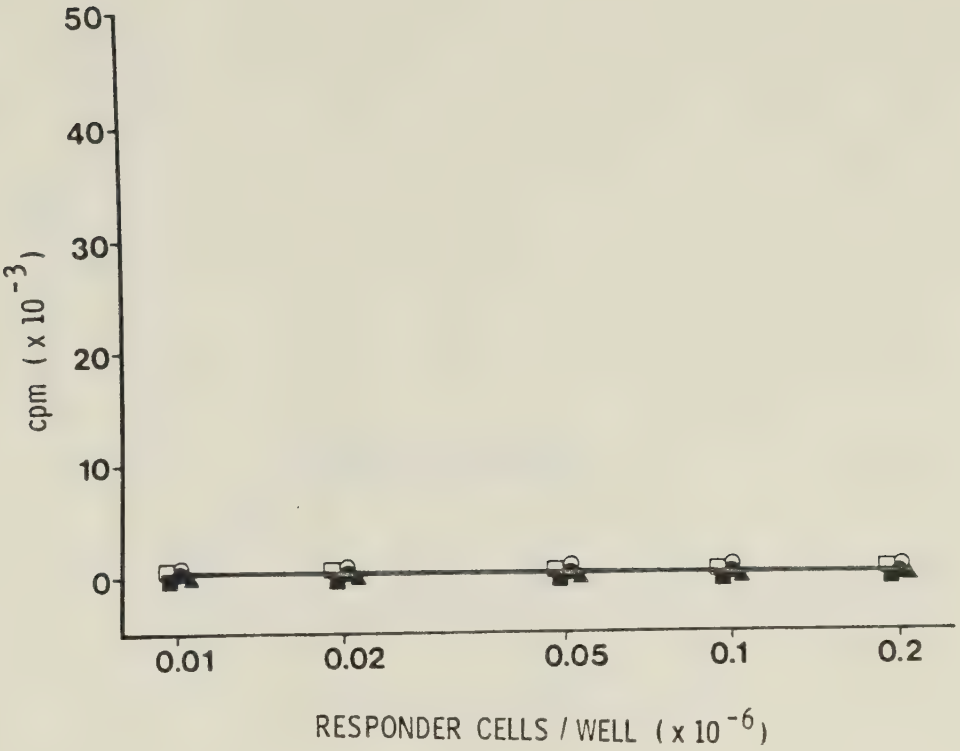

Figure 12. MLC of the <1.5 mm/hr sedimentation velocity fraction of primed spleen cells. Fractionated cells were challenged with various strains of stimulator cells ($1x10^6$/well) in 96 well trays for 3 days. At this time, cultures were pulsed with [^{3}H]-thymidine and reincubated for 20 h and harvested: ● - ● CBA/CaJ stimulators; Δ - Δ B10.T(6R) stimulators; □- □ C57BL/6 stimulators; o - o C3H.OH stimulators; ▲ - ▲ C3H.NB/Sn stimulators; ■- ■ C3H.HeJ stimulators.

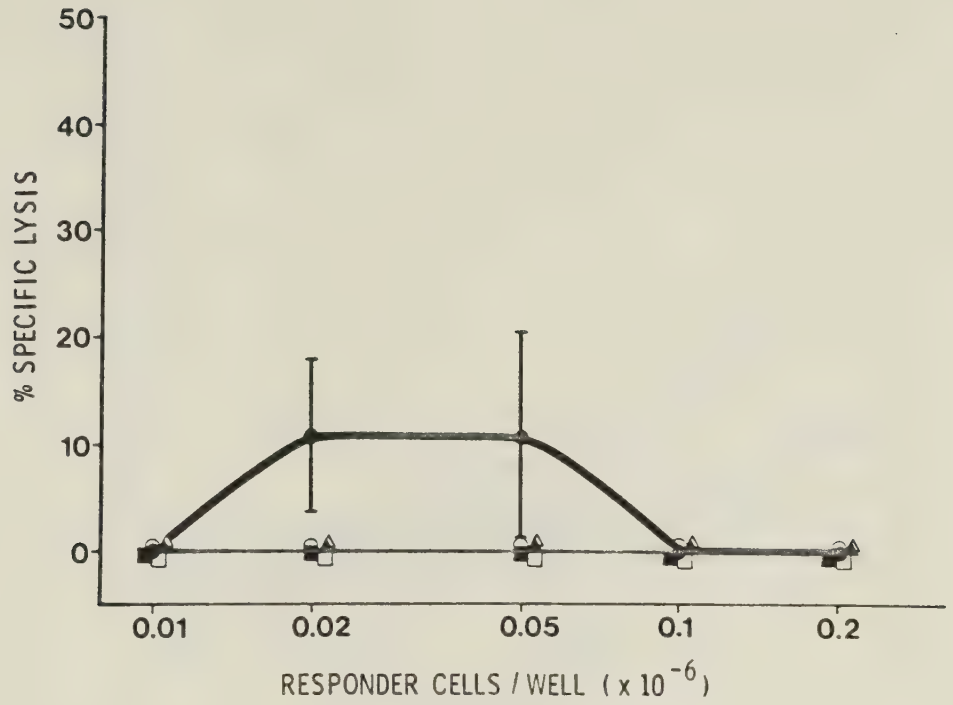

Figure 13. Cytotoxic response of the 1.5 - 5.0 mm/hr sedimentation velocity fraction of primed spleen cells. Fractionated cells were challenged with various strains of stimulator cells ($1x10^6$/well) in 96 well trays for 5 days, at which time an in situ cytotoxicity assay was done: ● - ● CBA/CaJ stimulators; Δ - Δ B10.T(6R) stimulators; □ - □ C57BL/6 stimulators; o - o C3H.OH stimulators; ▲ - ▲ C3H.NB/Sn stimulators; ■ - ■ C3H.HeJ stimulators.

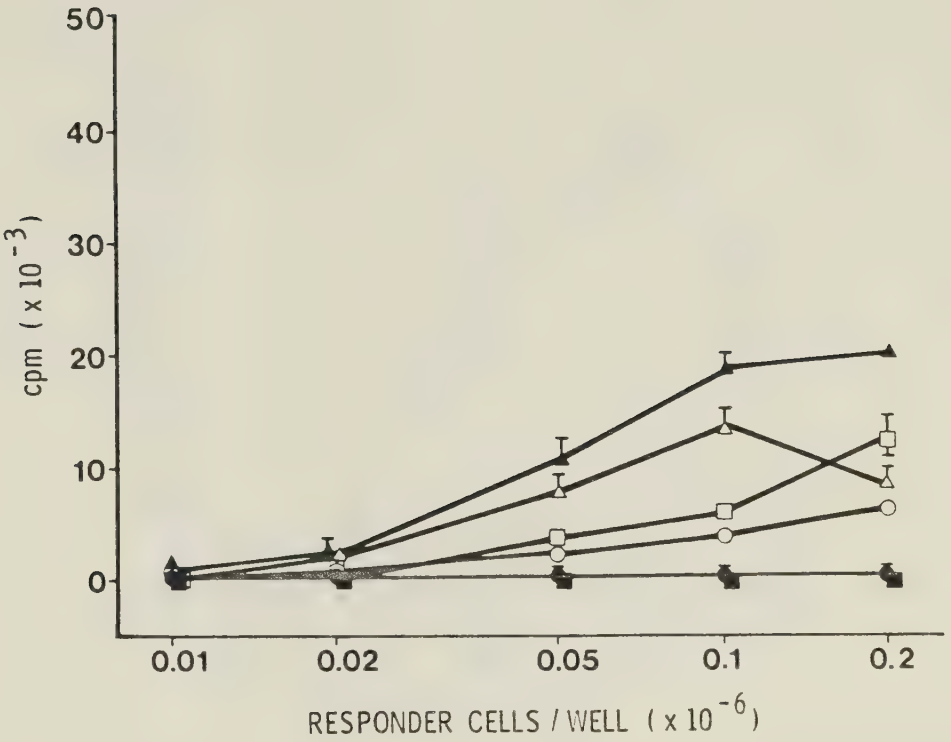

Figure 14. MLC of the 1.5 - 5.0 mm/hr sedimentation velocity fraction of primed spleen cells. Fractionated cells were challenged with various strains of stimulator cells ($1x10^6$/well) in 96 well trays for 3 days. At this time, cultures were pulsed with [^{3}H]-thymidine and reincubated for 20 h and harvested: ● - ● CBA/CaJ stimulators; Δ - Δ B10.T(6R) stimulators; □ - □ C57BL/6 stimulators; o - o C3H.OH stimulators; ▲ - ▲ C3H.NB/Sn stimulators; ■ - ■ C3H.HeJ stimulators.

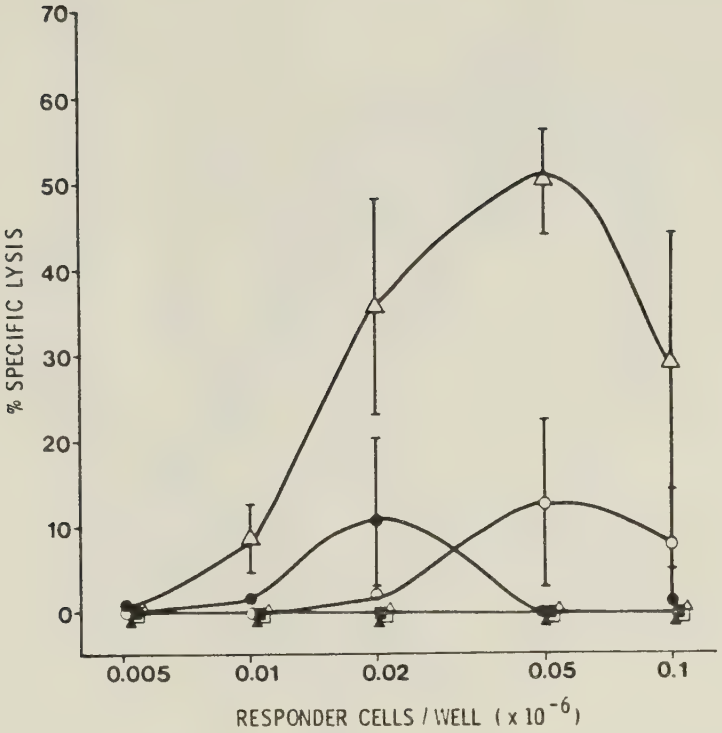

Figure 15. Cytotoxic response of the >5 mm/hr sedimentation velocity (blast) fraction of primed spleen cells. Fractionated cells were challenged with various strains of stimulator cells ($1x10^6$/well) in 96 well trays for 5 days, at which time an *in situ* cytotoxicity assay was done: ● - ● CBA/CaJ stimulators; Δ - Δ B10.T(6R) stimulators; □ - □ C57BL/6 stimulators; O - O C3H.OH stimulators; ▲ - ▲ C3H.NB/Sn stimulators; ■ - ■ C3H.HeJ stimulators.

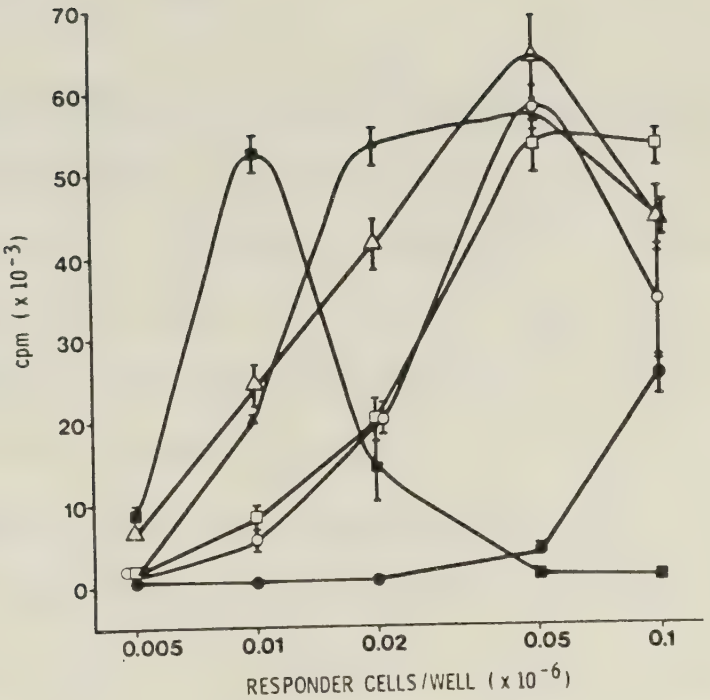

Figure 16. MLC of the >5.0 mm/hr sedimentation velocity (blast) fraction of primed spleen cells. Fractionated cells were challenged with various strains of stimulator cells ($1x10^6$/well) in 96 well trays for 3 days. At this time, cultures were pulsed with [^{3}H]-thymidine and reincubated for 20 h and harvested: ● - ● CBA/CaJ stimulators; Δ - Δ B10.T(6R) stimulators; □ - □ C57BL/6 stimulators; o - o C3H.OH stimulators; ▲ - ▲ C3H.NB/Sn stimulators; ■ - ■ C3H.HeJ stimulators.

ulation of specific alloantigen-reactive cells using the velocity sedimentation technique (78).

F. Clonal Enrichment by Restimulation of Alloantigen-reactive Cells

Unprimed B10 spleen cells develop cytotoxic activity and proliferate in MLC in response to a variety of stimulating allogeneic cells (Figures 17 and 18), as may be expected. B10(H-2^b) spleen cells primed to B10.D2 (H-2^d) stimulator cells for 7 days remained responsive to several strains of allogeneic cells in MLC (Figure 19).

As MLC activity returns to baseline levels by 7 days after primary stimulation (Figure 5), the responder B10 cells were restimulated by B10.D2 at this time. After a total of 14 days in MLR, a cytotoxicity assay was done. It resulted in cytotoxic activity specific for B10.D2 target cells (Figure 20). A significant but less marked cytotoxic response was also directed at B10.T(6R) target cells. This was expected, as B10.T(6R) cells are identical at the H-2 D region (Table III) with B10.D2 (the priming allogeneic cells).

Cell proliferation in MLC was found to be elicited only by B10.D2 stimulator cells (Figure 21). Significant stimulation by B10.T(6R) in MLC would not be expected as in the case of cytotoxic activity because the D region shared by the two strains is not, with few exceptions, a strong stimulus for cell proliferation (73,80).

This clonal enrichment technique was thus successful in generating alloantigen-reactive cells that respond to a single H-2 haplotype. In particular, as responder cell proliferation was seen only when stimulated by that haplotype, it could be assumed that the helper cells in this purified population of lymphocytes would generate a helper factor that would be ideal for the study of its specificity in modified Marbrook

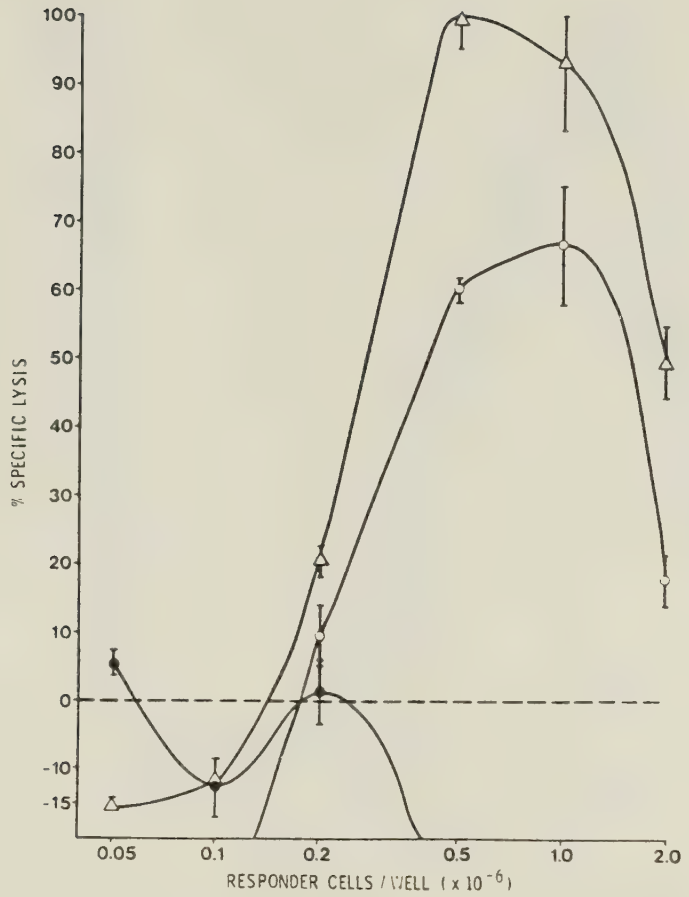

Figure 17. Cytotoxic response of unprimed B10 spleen cells. B10 spleen cells were stimulated by several strains of allogeneic spleen cells (1×10^6/well) in 96 well trays and incubated for 5 days, at which time an <u>in situ</u> cytotoxicity assay was performed: ● - ● B10 stimulators; Δ - Δ B10.D2 stimulators; □ - □ B10.AKM stimulators; 0 - 0 B10.T(6R) stimulators; 0 - 0 B10.T(6R) stimulators.

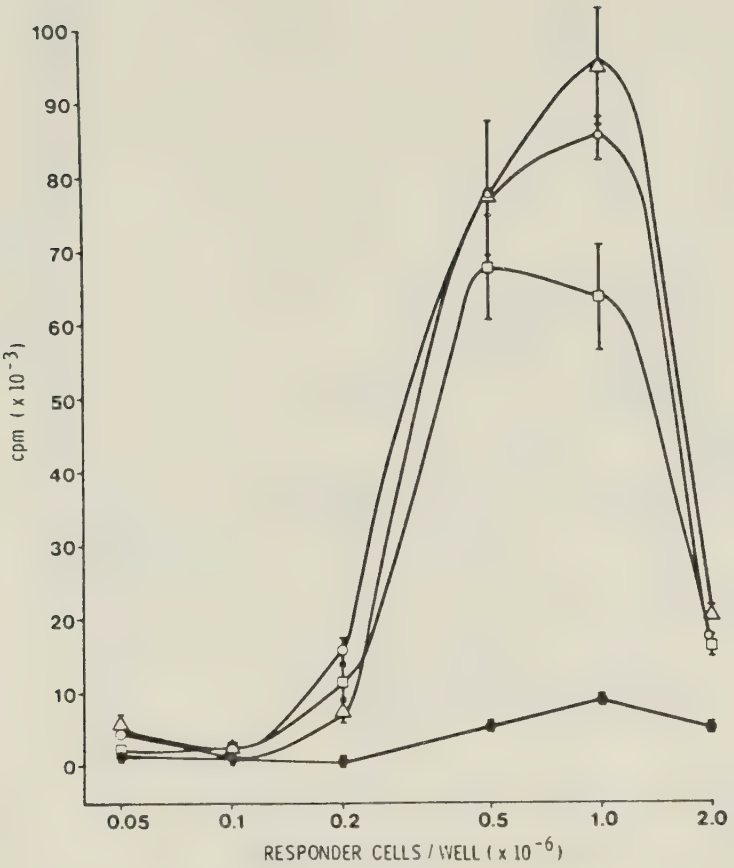

Figure 18. MLC of unprimed B10 spleen cells. B10 spleen cells were stimulated by several strains of allogeneic spleen cells ($1x10^6$/well) in 96 well trays and incubated for 3 days, at which time they were pulsed with [^{3}H]-thymidine. The cultures were reincubated for 20 h and harvested: ● - ● B10 stimulators; Δ - Δ B10.D2 stimulators; □ - □ B10.AKM stimulators; O - O B10.T(6R) stimulators.

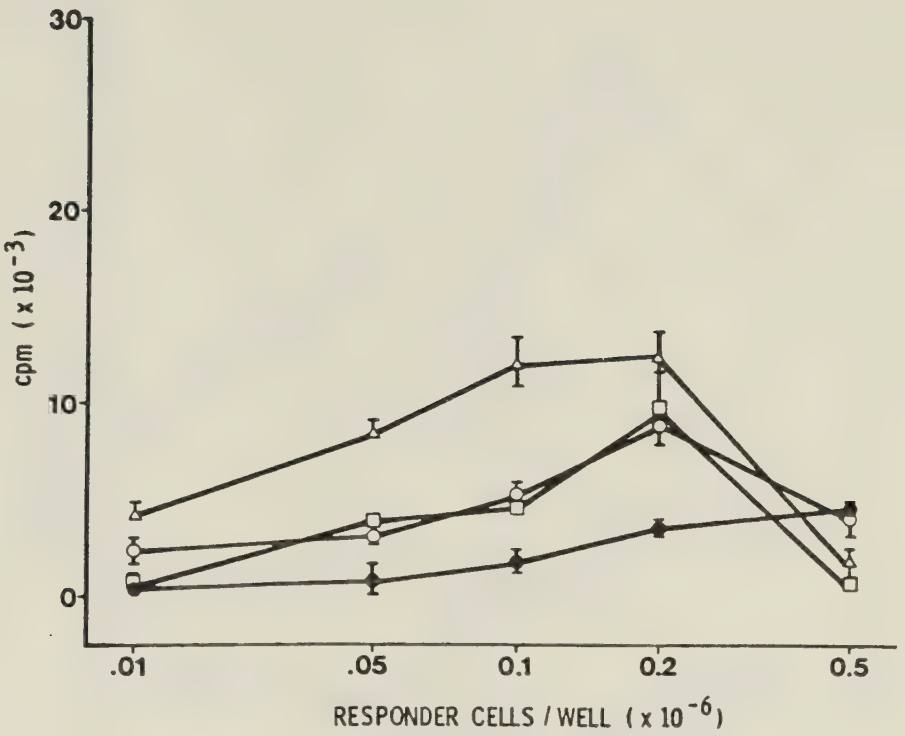

Figure 19. MLC of primed B10 spleen cells. B10 spleen cells ($4x10^6$/well) were primed to B10.D2 spleen cells ($4x10^6$/well) in 16 mm trays for 7 days. They were then harvested and secondary cultures were set up against several strains of allogeneic stimulator cells ($1x10^6$/well) in 96 well tissue culture trays. After 3 days of incubation, the cultures were pulsed with [^{3}H]-thymidine, reincubated for 20 h, and harvested: • - • B10 stimulators; Δ - Δ B10.D2 stimulators; □- □ B10.AKM stimulators; O - O B10.T(6R) stimulators.

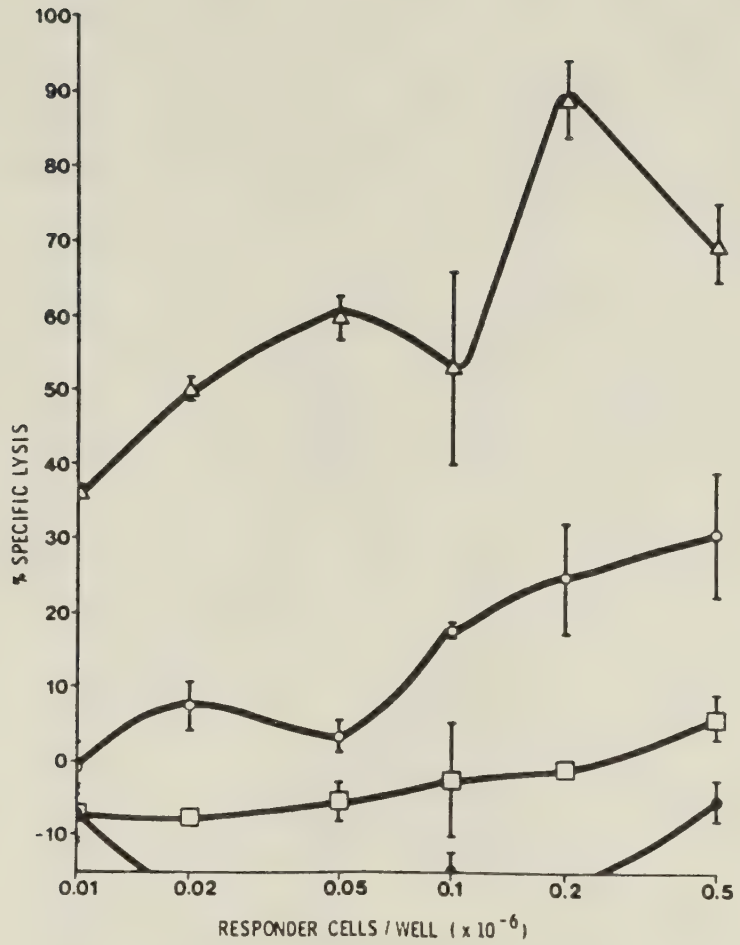

Figure 20. Cytotoxic response of double-primed B10 spleen cells. After 15 days in MLR, responder B10 spleen cells were harvested and secondary cultures were set up against several strains of allogeneic stimulator cells ($1x10^6$/well) in 96 well tissue culture trays. After 5 days of incubation, an in situ cytotoxicity assay was performed: ● - ● B10 stimulators; Δ - Δ B10.D2 stimulators; □ - □ B10.AKM stimulators; 0 - 0 B10.T(6R) stimulators. (The bimodal Δ - Δ curve probably represents an error in the $0.1x10^6$ responder cells/well dilution).

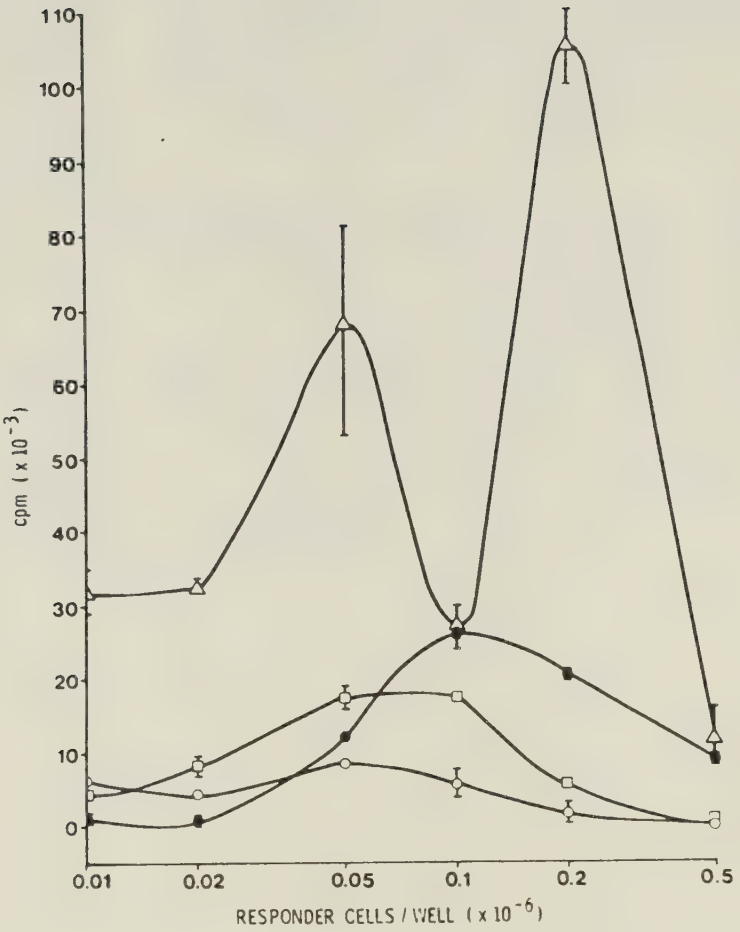

Figure 21. MLC of double-primed B10 spleen cells. After 14 days in MLR, responder B10 spleen cells were harvested and secondary cultures were set up against several strains of allogeneic stimulator cells ($1x10^6$/well) in 96 well trays. After 3 days of incubation, the cultures were pulsed with [^{3}H]-thymidine and reincubated for 20 h, at which time they were harvested: ● - ● B10 stimulators; Δ - Δ B10.D2 stimulators; □ - □ B10.AKM stimulators; O - O B10.T(6R) stimulators. (The bimodal Δ - Δ curve probably represents an error in the $0.1x10^6$ responder cells/well dilution.)

double chamber cultures. If helper factor which augments the cytotoxic response is genetically restricted, it would cooperate with only syngeneic responder cells. If it is alloantigen-specific, it would cooperate with cytotoxic cells against only the stimulator cells to which the helper cells were primed.

However, multiple attempts at producing helper activity in the modified Marbrook culture system using the clonally enriched helper cells failed to demonstrate any consistent helper effect and time did not permit a thorough investigation of the antigen specificity of helper factor generated in this manner.

Chapter IV. Discussion

The properties of helper cells generated in the system described here are similar to those described by others, i.e., they bear the Thy 1 antigen (61,62) and are activated mainly by I-A subregion differences in MLR (78-80). However, unlike the findings by other authors, the helper cells in this study could also be generated in response to H-2 subregion differences other than I-A (Figure 4), although to a lesser degree.

In agreement with reports of others, the helper factor generated by these helper cells appears not to be genetically restricted (62,108), and is not alloantigen specific (110-112) in the cytotoxic T cell response. There are at least two possibilities to explain these findings. First, an alloantigen nonspecific factor may have been produced by a single clone of helper cells. Secondly, the results may be invalid because 2-mercaptoethanol (2-ME) was present as a supplement in the medium used for the generation of helper cells. 2-ME has many known effects on immune responsiveness which include enhancement of lymphocyte survival in tissue culture (121). Another known effect is that of polyclonal activation of T cells (121). Thus, it is conceivable that the 2-ME may have stimulated the activation of multiple clones of helper T cells of multiple specificities. This would result in the generation of factors from clones of different specificities and lead to the observation that the described helper factor is not alloantigen specific.

Reports of helper factor specificity by other authors are contradictory. Results obtained depend on the experimental system used to demonstrate helper activity and the class of immune response studied (e.g. antibody-forming or cytotoxic T cell response). It is possible that the apparently conflicting results are due to descriptions of

different helper factors (122). There is growing evidence that helper factors of different specificities are generated by different subpopulations of helper cells that augment the B cell response (122-124). It appears that the conditions that control whether the specific or nonspecific factor is generated include the nature and the dose of the stimulating antigen (123,124). It also appears that the specific helper factor is generated by Ia antigen-negative cells while the nonspecific factor-generating cells are Ia antigen-positive (122).

To date, there is no evidence to suggest that helper cells augmenting T cell-mediated responses can be similarly classified into subpopulations which generate antigen specific and nonspecific helper factor. With one exception (109), all reports of helper factor activity in cytotoxic T cell responses have shown that it is not specific to the priming allogeneic cells. But, apart from the exception mentioned (109), there is other evidence to suggest the possibility of an alloantigen specific factor. A histocompatibility (H) alloantigen specific helper factor that augments the B cell response has been generated in MLR (95). It may be possible that the same factor that augments the B cell response also augments effector T cell responses. It may also be possible to generate a specific factor to cooperate in the cytotoxic T cell response if similar conditions to those used by these investigators are employed. The conditions include i) omission of 2-ME from culture media used to generate helper cells and ii) helper cell generation by enrichment for specific alloantigen-reactive cells. It was attempted to duplicate these conditions in this study.

Several interesting results were obtained. Firstly, it was observed that, of the cell size fractionated groups, only the blast cell fraction responded in the MLC and cytotoxicity assays (Figure 11-16). Similar

results have been previously reported (78). It would be anticipated that the smaller fractions of cells should include responding cells with specificities for alloantigen determinants other than those of the priming stimulator cells. As they did not respond at all, it appears that the smaller cell fractions are totally depleted of MLR-reactive T cells (78).

Another unexpected finding is that the blast cells proliferate in response to several allogeneic H-2 haplotypes (Figure 16) (78), yet cytotoxicity is specific for the priming allogeneic target cells (Figure 15). A possible explanation for this observation is that the determinants to which cells respond in these assays may be different (126). Therefore, the determinants on various H-2 haplotypes recognized by cytotoxic T cells may not be as cross-reactive as the determinants which stimulate cell proliferation in MLC (78).

However, this explanation does not appear to concur with the results found in the MLC responses of the double-primed cells (Figure 21). Here, there is no cross-reactivity. However, a hypothesis to reconcile these differences in specificity of MLC responses may be made if it is assumed that the determinants which induce cell proliferation are of different strengths. Thus, in a primary MLC with fresh responder cells, both strong and weak determinants can induce cell proliferation. However, when MLR is prolonged, the cells responding to weak determinants may proliferate at a slower rate than those responding to strong determinants and thus become "diluted" by specific strong determinant-reactive cells, or they may not be stimulated adequately to survive long-term culture. The end result after prolonged MLR would be cells responding specifically in MLC.

Despite the fact that the specific alloantigen-reactive cells that were isolated by restimulation in MLR demonstrated inconsistent helper activity in this short study, this technique for helper cell generation may be useful for future investigation of helper factor specificity. As the H-2 complex contains enough DNA to code for large numbers of determinants, the alloantigen-reactive cells generated in response to whole H-2 complex differences may contain a very heterogeneous population of monospecific helper cells (78). But if appropriate culture conditions to enrich for cells responding to whole H-2 complex incompatibilities could be established, it may be possible to use these conditions to generate helper cells in response to only small portions of the H-2 complex (78). The number of possible monospecific helper cell populations in these cells would therefore be diminished and it may be possible to study the specificity of helper factor generated by a clone of helper cells.

Bibliography

1. Humphrey, J.H., and White, R.G. 1970. Immunology for Students of Medicine, Third Edition, Blackwell Scientific Publications.

2. Roitt, I.M. 1974. Essential Immunology, Second Edition, Blackwell Scientific Publications.

3. Shortman, K., Fidler, J.M., Schlegel, R.A., Nossal, G.J.V., Howard, M., Lipp, J., and von Boehmer, H. 1976. Subpopulations of B lymphocytes: Physical separation of functionally distinct stages of B cell differentiation. Contemporary Topics in Immunobiology, Vol. 5, p 1-45. W.O. Weigle, ed., Plenum Press, New York.

4. Szenberg, A., and Warner, N.L. 1962. Immunological function of thymus and Bursa of Fabricus. Dissociation of immunological responsiveness in fowls with a hormonally arrested development of lymphoid tissues. Nature 194:146.

5. Claman, H.N., Chaperon, E.A., and Triplett, R.F. 1966. Immunocompetence of transferred thymus-marrow cell combinations. J. Immunol. 97:828.

6. Mitchell, G.F., and Miller, J.F.A.P. 1968. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. J. Exp. Med. 128:821.

7. Claman, H.M., and Chaperon, E.A. 1969. Immunological complementation between thymus and marrow cells - a model for the two-cell theory of immunocompetence. Transplant. Rev. 1:92.

8. Burnet, F.M., and Holmes, M.C. 1962. Immunological function of thymus and Bursa of Fabricus. Thymus lesions in an autoimmune disease of mice. Nature 194:146.

9. Miller, J.F.A.P., Mitchell, G.F., and Weiss, N.S. 1967. Cellular basis of the immunological defects in thymectomized mice. Nature 214:992.

10. Miller, J.F.A.P., and Mitchell, G.F. 1969. Thymus and antigen-reactive cells. Transplant. Rev. 1:3.

11. Stutman, O.E., Yunis, E.J., and Good, R.A. 1968. Carcinogen-induced tumors of the thymus. I. Restoration of neonatally thymectomized mice with a functional thymoma. J. Nat. Cancer Inst. 41:1431.

12. Davies, A.J.S., Leuchars, E., Wallis, V., Marchant, R., and Elliott, E.V. 1967. The failure of thymus-derived cells to make antibody. Transplantation 5:222.

13. Roitt, I.M., Greaves, M.F., Torrigianni, G., Brostoff, J., and Playfair, J.H.L. 1969. The cellular basis of immunological responses. Lancet II:367.

14. Raff, M.C. 1970. Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. Immunology 19:637.

15. Cantor, H., and Boyse, E.A. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T cell subclasses is a differentiative event independent of antigen. J. Exp. Med. 141:1376.

16. Cantor, H., and Boyse, E.A. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly+ cells in the generation of killer activity. J. Exp. Med. 141:1390.

17. Greaves, M., and Janossy, G. 1972. Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transplant. Rev. 11:87.

18. Raff, M.C., and Wortis, H.H. 1970. Thymus dependence of θ-bearing cells in the peripheral lymphoid tissues of mice. Immunology 18:931.

19. Katz, D.H., and Benacerraf, B. 1972. The regulatory influence of activated T cells on B cell responses to antigen. Adv. Immunol. 15:1.

20. Oppenheim, J.J. 1968. Relationship of *in vitro* lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. Fed. Proc. 27:21.

21. Stutman, O., Yunis, E.J., and Good, R.A. 1970. Cooperative effect of thymic function and lymphohemopoietic cells in restoration of neonatally thymectomized mice. J. Exp. Med. 132:583.

22. Cerottini, J-C., Nordin, A.A., and Brunner, K.T. 1970. *In vitro* cytotoxic activity of thymus cells sensitized to alloantigens. Nature 227:72.

23. Wagner, H. 1971. Cell mediated immune response *in vitro*: independent differentiation of thymocytes into cytotoxic lymphocytes. Eur. J. Immunol. 1:498.

24. Cerottini, J-C., and Brunner, K.T. 1974. Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. Adv. Immunol. 18:67.

25. Miller, J.F.A.P., Brunner, K.T., Sprent, S., Russel, P.J., and Mitchell, G.F. 1971. Thymus-derived cells as killer cells in cell-mediated immunity. Transplant. Proc. 3:915.

26. Cantor, H., and Asofsky, R. 1970. Synergy among lymphoid cells mediating the graft-vs-host response. II. Synergy in graft-vs-host reactions produced by Balb/c lymphoid cells of differing anatomical origin. J. Exp. Med. 131:235.

27. Cantor, H., Mandel, M., and Asofsky, R. 1970. Studies of thoracic duct lymphocytes of mice. II. A quantitative comparison of the capacity of thoracic duct lymphocytes and other lymphoid cells to induce graft-vs-host reactions. J. Immunol. 104:409.

28. Cantor, H., and Asofsky, R. 1972. Synergy among lymphoid cells mediating the graft-vs-host response. III. Evidence for interaction between two types of thymus-derived cells. J. Exp. Med. 135:764.

29. Miller, J.F.A.P., and Mitchell, G.F. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. J. Exp. Med. 128:801.

30. Martin, W.J., and Miller, J.F.A.P. 1968. Cell to cell interaction in the immune response. IV. Site of action of anti-lymphocyte globulin. J. Exp. Med. 128:855.

31. Wagner, H. 1973. Synergy during in vitro cytotoxic allograft responses. I. Evidence for cell interaction between thymocytes and peripheral T cells. J. Exp. Med. 138:1379.

32. Häyry, P., and Andersson, L.C. 1974. T cell synergy in mixed lymphocyte culture-induced cytolysis. Eur. J. Immunol. 4:145.

33. Dutton, R.W., and Eady, J.D. 1964. An in vitro system for the study of the mechanism of antigenic stimulation in the secondary response. Immunology 7:40.

34. Häyry, P., and Defendi, V. 1970. Mixed lymphocyte cultures produce effector cells: model in vitro for allograft rejection. Science 168:133.

35. Takiguchi, T., Adler, W.H., and Smith, R.T. 1971. Cellular recognition in vitro by mouse lymphocytes. Effects of neonatal thymectomy and thymus graft restoration on alloantigen and PHA stimulation of whole and gradient-separated subpopulations of spleen cells. J. Exp. Med. 133:63.

36. Stobo, J.D., Paul, W.E., and Henny, C.S. 1973. Functional heterogeneity of murine lymphoid cells. IV. Allogeneic mixed lymphocyte reactivity and cytolytic activity as functions of distinct T cell subsets. J. Immunol. 110:652.

37. Corley, R.B., and Kindred, B. 1977. In vivo responses of alloreactive lymphocytes stimulated in vitro. Helper cell activity of MLR-primed lymphocytes. Scand. J. Immunol. 6:923.

38. Corley, R.B. and Kindred, B. 1977. In vivo responses of alloreactive lymphocytes stimulated in vitro. Skin graft rejection mediated by MLR-primed lymphocytes. Scand. J. Immunol. 6:991.

39. Gershon, R.K., and Kondo, K. 1970. Cell interactions in the induction of tolerance. Immunology 18:723.

40. Dutton, R.W. 1972. Inhibitory and stimulatory effects of Concanavalin A on the response of mouse spleen cell suspensions to antigen. I. Characterization of the inhibitory cell activity. J. Exp. Med. 136:1445.

41. Dutton, R.W. 1973. Inhibitory and stimulatory effects of Concanavalin A on the response of mouse spleen cell suspensions to antigen. II. Evidence for separate stimulatory and inhibitory cells. J. Exp. Med. 138:1496.

42. Chess, L., and Schlossman, S.F. 1977. Human lymphocyte subpopulations. Adv. Immunol. 25:213.

43. Kiessling, R., Klein, E., and Wigzell, H. 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur. J. Immunol. 5:112.

44. Kiessling, R., Klein, E., Pross, H., and Wigzell, H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. Eur. J. Immunol. 5:117.

45. Pross, H.F., and Baines, M.G. 1977. Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells. VI. A brief review. Cancer Immunol. Immunother. 3:75.

46. Trinchieri, G., Bauman, P., deMarchi, M., and Tökés, Z. 1975. Antibody-dependent cell-mediated cytotoxicity in humans. I. Characterization of the effector cell. J. Immunol. 115:249.

47. Andersson, J., and Blomgren, H. 1971. Evidence for T-independent humoral antibody production against polyvinylpyrrolidone and *E. coli* lipopolysaccharide. Cell. Immunol. 2:411.

48. Miranda, J.J. 1972. Studies on immunological paralysis. IX. The immunogenicity of levan in mice. Immunology 23:829.

49. Tigelaar, R.E., and Feldman, M. 1973. Synergy among thymocytes and peripheral lymph node cells in the *in vitro* generation of lymphocytes cytotoxic to alloantigens. Transplant. Proc. 4:1711.

50. Mosier, D.E. 1967. A requirement for two cell types for antibody formation *in vitro*. Science 158:1573.

51. Hersh, E.M., and Harris, J.E. 1968. Macrophage-lymphocyte interaction in the antigen-induced blastogenic response of human peripheral blood leukocytes. J. Immunol. 100:1184.

52. Shortman, K., Diener, E., Russell, P., and Armstrong, W.D. 1970. The role of nonlymphoid accessory cells in the immune response to different antigens. J. Exp. Med. 131:461.

53. Wagner, H., Feldman, M., Schrader, J.W., and Boyle, W. 1972. Cell-mediated immunity in vitro. III. The requirement for macrophages in cytotoxic reactions against cell-bound and subcellular alloantigens. J. Exp. Med. 136:331.

54. Wagner, H., Rollinghoff, M., and Nossal, G.J.V. 1973. T cell-mediated immune responses induced in vitro: A probe for allograft and tumor immunity. Transplant. Rev. 17:3-36.

55. Lee, K-C., Shiozawa, C., Shaw, A., and Diener, E. 1976. Requirement for accessory cells in the antibody response to T cell-independent antigens in vitro. Eur. J. Immunol. 6:63.

56. Waldron, J.A., Horn, R.G., and Rosenthal, A.S. 1973. Antigen-induced proliferation of guinea pig lymphocytes in vitro: Obligatory role of macrophages in the recognition of antigen by immune T lymphocytes. J. Immunol. 111:58.

57. Rosenstreich, D.L., and Rosenthal, A.S. 1974. Peritoneal exudate lymphocyte. III. Dissociation of antigen-reactive lymphocytes from antigen-binding cells in a T lymphocyte-enriched population in the guinea pig. J. Immunol. 112:1085.

58. Ehrenreich, B.A., and Cohn, Z.A. 1967. The uptake and digestion of iodinated human serum albumin by macrophages in vitro. J. Exp. Med. 126:941.

59. Ellner, J.J., and Rosenthal, A.S. 1975. Quantitation and immunologic aspects of the handling of 2,4-dinitrophenyl guinea pig albumin by macrophages. J. Immunol. 114:1563.

60. Rosenthal, A.S., and Shevach, E.M. 1973. The function of macrophages in antigen recognition by guinea pig T lymphocytes. I. The requirement for histocompatible macrophages and lymphocytes. J. Exp. Med. 138:1194.

61. Schimpel, A., and Wecker, E. 1972. Replacement of T cell function by a T cell product. Nature (New Biol.) 237:15.

62. Pilarski, L.M. 1977. A requirement for antigen-specific helper T cells in the generation of cytotoxic T cells from thymocyte precursors. J. Exp. Med. 145:709.

63. Pickel, K., Hammerling, U., and Hoffmann, M.K. 1976. Ly phenotype of T cells releasing T cell replacing factor. Nature 264:72.

64. Tokuhisa, T., Taniguchi, M., Okumura, K., and Tada, T. 1978. An antigen-specific I region gene product that augments the antibody response. J. Immunol. 120:414.

65. Okumura, K., Herzenberg, L.A., Murphy, D.B., McDevitt, H.O., and Herzenberg, L.A. 1976. Selective expression of H-2 (I region) loci controlling determinants on helper and suppressor T lymphocytes. J. Exp. Med. 144:685.

66. McDevitt, H.O., Delovitch, T.L., Press, J.L., and Murphy, D.B. 1976. Genetic and functional analysis of the Ia antigens: Their possible role in regulating the immune response. Transplant Rev. 30:197.

67. Andersson, B., and Blomgren, H. 1970. Evidence for a small pool of immunocompetent cells in the mouse thymus. Its role in the humoral antibody response against sheep erythrocytes, bovine serum albumin, ovalbumin and the NIP determinant. Cell. Immunol. 1:362.

68. Blomgren, H., and Andersson, B. 1970. Characteristics of the immunocompetent cells in the mouse thymus: cell population changes during cortisone-induced atrophy and subsequent regeneration. Cell. Immunol. 1:545.

69. Lee, K-C. 1977. Cortisone as a probe for cell interactions in the generation of cytotoxic T cells. I. Effect on helper cells, cytotoxic T cell precursors, and accessory cells. J. Immunol. 119:1836.

70. Katz, D.H., Paul, W.E., Goidl, E.A., and Benacerraf, B. 1970. Radioresistance of cooperative function of carrier-specific lymphocytes in antihapten antibody responses. Science 170:462.

71. Benacerraf, B., and McDevitt, H.O. 1972. Histocompatibility-linked immune response genes. A new class of genes that controls the formation of specific immune responses has been identified. Science 175:273.

72. Klein, J. 1975. Biology of the Mouse Histocompatibility-2 Complex. Springer-Verlag, New York.

73. Klein, J. 1976. An attempt at an interpretation of the mouse H-2 complex. In: Contemporary Topics in Immunobiology. Vol. 5. W.O. Weigle, ed., Plenum Press, New York.

74. Hämmerling, G.J. 1976. Tissue distribution of Ia antigens and their expression on lymphocyte subpopulations. Transplant. Rev. 30:64.

75. Niederhuber, J.E., and Frelinger, J.A. 1976. Expression of Ia antigens on T and B cells and their relationship to immune response functions. Transplant. Rev. 30:101.

76. Klein, J., and Hauptfeld, V. 1976. Ia antigens: Their serology, molecular relationships, and their role in allograft reactions. Transplant. Rev. 30:83.

77. Schwartz, R.H., Dorf, M.E., Benacerraf, B., and Paul, W.E. 1976. The requirement for two complementing Ir-GLϕ immune response genes in the T lymphocyte proliferative response to Poly-($Glu^{33}Lys^{36}Phe^{11}$). J. Exp. Med. 143:897.

78. Peck, A.B., Wigzell, H., Janeway, C., and Andersson, L.C. 1977. Environmental and genetic control of T cell activation in vitro: A study using isolated alloantigen-activated T cell clones. Immunol. Rev. 35:146.

79. Shreffler, D.C., and David, C.S. 1975. The H-2 major histocompatibility complex and the I immune response region: genetic variation, function and organization. Adv. Immunol. 20:125.

80. Bach, F.H., Bach, M.L., and Sondel, P.M. 1976. Differential function of major histocompatibility complex antigens in T lymphocyte activation. Nature 259:273.

81. Katz, D.H., Hamaoka, T., Dorf, M.E., Maurer, P.H., and Benacerraf, B. 1973. Cell interactions between histoincompatible T and B lymphocytes. IV. Involvement of the immune response (Ir) gene in the control of lymphocyte interactions in the responses controlled by the gene. J. Exp. Med. 138:734.

82. Katz, D.H., and Benacerraf, B. 1975. The function and interrelationships of T cell receptors, Ir genes and other histocompatibility gene products. Transplant. Rev. 22:175.

83. Munro, A.J., and Taussig, M.J. 1975. Two genes in the major histocompatibility complex control of immune response. Nature 256:103.

84. Feldman, M., and Basten, A. 1972. Cell interactions in the immune response in vitro. III. Specific collaboration across a cell impermeable membrane. J. Exp. Med. 136:49.

85. Waldman, H., and Munro, A. 1973. T cell-dependent mediator in the immune response. Nature 243:356.

86. Gorczynski, R.M., Miller, R.G., and Phillips, R.A. 1973. Reconstitution of T-cell-depleted spleen cell populations by factors derived from T cells. I. Conditions for the production of active T cell supernatants. J. Immunol. 110:968.

87. Taussig, M.J. 1974. T cell factor which can replace T cells in vivo. Nature 248:234.

88. Shiozawa, C., Singh, B., Rubinstein, S., and Diener, E. 1977. Molecular control of B cell triggering by antigen-specific T cell-derived helper factor. J. Immunol. 118:2199.

89. Feldman, M., and Basten, A. 1972. Cell interactions in the immune response in vitro. IV. Comparison of the effects of antigen-specific and allogeneic thymus-derived cell factors. J. Exp. Med. 136:722.

90. Rubin, A.S., and Coons, A.H. 1972. Specific heterologous enhancement of immune responses. III. Partial characterization of supernatant material with enhancing activity. J. Immunol. 108:1597.

91. Gorczynski, R.M., Miller, R.G., and Phillips, R.I. 1973. Reconstitution of T cell-depleted spleen cell populations by factors derived from T cells. III. Mechanism of action of T cell derived factors. J. Immunol. 111:901.

92. Watson, J.D. 1973. The role of humoral factors in the initiation of in vitro primary immune responses. III. Characterization of factors that replace thymus-derived cells. J. Immunol. 111:1301.

93. Waldman, H., and Munro, A. 1974. T cell-dependent mediator in the immune response. II. Physical and biological properties. Immunology 27:53.

94. Hunter, P., and Kettman, J.R. 1974. Mode of action of a supernatant activity from T cell cultures that nonspecifically stimulates the humoral immune response. Proc. Natl. Acad. Sci. 71:512.

95. Kindred, B., and Corley, R.B. 1977. A T cell-replacing factor specific for histocompatibility antigens in mice. Nature 268:531.

96. Taussig, M.J., and Munro, A.J. 1974. Removal of specific cooperative T cell factor by anti-H-2 but not by anti-Ig sera. Nature 251:63.

97. McDougal, J.S., Cort, S.P., and Gordon, D.S. 1977. Generation of T helper cells in vitro. III. Helper cell culture-derived factors are related to alloantigens coded for by the I region of the H-2 major histocompatibility complex. J. Immunol. 119:1933.

98. Amerding, D., and Katz, D.H. 1974. Activation of T and B lymphocytes in vitro. II. Biological and biochemical properties of an allogeneic effect factor (AEF) active in triggering specific B lymphocytes. J. Exp. Med. 140:19.

99. Amerding, D., Sachs, D.H., and Katz, D.H. 1974. Activation of T and B lymphocytes in vitro. III. Presence of Ia determinants on allogeneic effect factor. J. Exp. Med. 140:1717.

100. Amerding, D., Kuho, R.T., Grey, H.M., and Katz, D.H. 1975. Activation of T and B lymphocytes in vitro: presence of β_2-microglobulin determinants on allogeneic effect factor. Proc. Nat. Acad. Sci. (Wash.) 72:4577.

101. Kennedy, J.C., Treadwell, P.E., and Lennox, E.S. 1970. Antigen-specific synergism in the immune response of irradiated mice given marrow cells and peritoneal cavity cells or extracts. J. Exp. Med. 132:353.

102. Howie, S., and Feldman, M. 1977. In vitro studies on H-2-linked unresponsiveness to synthetic polypeptides. III. Production of an antigen-specific T helper cell factor to (T,G)-A--L. Eur. J. Immunol. 7:417.

103. McDougal, J.S., and Gordon, D.S. 1977. Generation of T helper cells in vitro. I. Cellular and antigen requirements. J. Exp. Med. 145:676.

104. Dutton, R.W., Falkoff, R., Hirst, J.A., Hoffman, M., Kappler, J.W., Kettman, J.R., Lesley, J.F., and Vann, D. 1971. Is their evidence for a non-antigen specific diffusable chemical mediator from the thymus-derived cell in the initiation of the immune response? Progress in Immunology, p.355. B. Amos, ed., Academic Press, New York.

105. Ekpaha-Mensah, A., and Kennedy, J.C. 1971. New indicator of histocompatibility differences in vitro. Nature (New Biol.) 233:174.

106. Hunter, P., and Kettman, J.R. 1974. Mode of action of a supernatant activity from T cell cultures that nonspecifically stimulates the humoral immune response. Proc. Nat. Acad. Sci. 71:512.

107. Marwell, L., Kappler, J.W., and Marrack, P.C. 1976. Antigen-specific and nonspecific mediators of T cell/B cell cooperation. III. Characterization of the nonspecific mediator(s) from different sources. J. Immunol. 116:1379.

108. Altman, A., and Cohen, I.R. 1975. Cell-free media of mixed lymphocyte cultures augmenting sensitization in vitro of mouse T lymphocytes against allogeneic fibroblasts. Eur. J. Immunol. 5:437.

109. Baum, L.L, and Pilarski, L.M. 1978. In vitro generation of antigen-specific helper T cells which collaborate with cytotoxic T cell precursors. Manuscript in preparation.

110. Altman, A., and Cohen, I.R. 1974. The nonspecific helper effect of mixed lymphocyte reactions on the induction of T cell-mediated immunity in vitro. Eur. J. Immunol. 4:577.

111. Plate, J.M.D. 1976. Soluble factors substitute for T-T cell cooperation in generation of T-killer lymphocytes. Nature 260:329.

112. Orosz, C.G., and Finke, J.H. 1978. Influence of killer assisting factor (KAF) on generation of cytotoxic T cells. Cell. Immunol. 37:86.

113. Miller, R.G., and Phillips, R.A. 1969. Separation of cells by velocity sedimentation. J. Cell. Physiol. 73:191.

114. Andersson, L.C., and Häyry, P. 1975. Clonal isolation of allo-antigen-reactive T cells and characterization of their memory function. Transplant. Rev. 25:121.

115. Häyry, P., and Andersson, L.C. 1975. Sequential responses of mouse spleen T cells in mixed lymphocyte culture-induced cytolysis. J. Exp. Med. 141:508.

116. Häyry, P., and Andersson, L.C. 1974. Generation of T memory cells in one-way mixed lymphocyte culture. II. Anamnestic responses of 'secondary' lymphocytes. Scand. J. Immunol. 3:823.

117. Dennert, G., and Raschke, W. 1977. Continuously proliferating allospecific T cells, lifespan and antigen receptors. Eur. J. Immunol. 7:352.

118. Lee, K-C., and Berry, D. 1977. Functional heterogeneity in macrophages activated by Cornybacterium parvum: Characterization of subpopulations with different activities in promoting immune responses and suppressing tumor cell growth. J. Immunol. 118:1530.

119. Brunner, R.T., Mauel, J., Cerottini, J-C., and Chapuis, B. 1970. Studies of allograft immunity in mice. I. Induction, development and in vitro assay of cellular immunity. Immunology 18:501.

120. Armstrong, W.D., and Kraft, N.E. 1973. The early response of immunocompetent cells as analyzed by velocity sedimentation separation. J. Immunol. 110:157.

121. Chen, C., and Hirsch, J.G. 1972. The effects of mercaptoethanol and of peritoneal macrophages on the antibody-forming capacity of nonadherent mouse spleen cells in vitro. J. Exp. Medl. 136:604.

122. Tada, T., Takemori, T., Okumura, K., Nonaka, M., and Tokuhisa, T. 1978. Two distinct types of helper T cells involved in the secondary antibody response: independent and synergistic effects of Ia^- and Ia^+ helper T cells. J. Exp. Med. 147:446.

123. Marrack (Hunter), P.C., and Kappler, J.W. 1975. Antigen-specific and nonspecific mediators of T cell/Bcell cooperation. I. Evidence for their production by different cells. J. Immunol. 114:1116.

124. Marrack, P.C., and Kappler, J.W. 1976. Antigen-specific and non-specific mediators of T cell/B cell coperation. II. Two helper cells distinguished by their antigen sensitivities. J. Immunol. 116:1373.

125. Peck, A.B., Alter, B.J., Lindahl, K.F. 1976. Specificity in T cell-mediated lympholysis: Identical genetic control of the proliferative and effector phases of allogeneic and xenogeneic reactions. Transplant. Rev. 29:189.

126. Bach, F.H., Widmer, M.B., Bach, M.L., and Klein, J. 1972. Serologically-defined and lymphocyte-defined components of the major histocompatibility complex in the mouse. J. Exp. Med. 136:1430.